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Uterine Cancer in Connecticut: Late Deaths Among 5-Year Survivors [1, 2]

JOHN C. BAILAR III, *Field Investigator, Biometry Branch, National Cancer Institute,* [3] *Bethesda, Maryland*

SUMMARY

Long-term survival of 3,173 patients with cancer of the uterine cervix and 2,394 patients with cancer of the uterine corpus is compared with survival in the general population to get relative rates. Ratios of observed-to-expected survival rates, called relative survival rates, are given for periods up to 20 years after diagnosis and are analyzed with respect to age at diagnosis, stage of disease, treatment, and other variables. For each site, relative survival rates were low at first but rose to about 97 percent per year after 5 years and showed no further improvement. The effects of age and stage on relative survival rates were small after the first few years. After the 5th year, relative survival of patients with cervical tumors was the same whether the original treatment was radiation, surgery, or radiation plus surgery. The group of corpus-cancer patients treated with surgery and the group treated with radiation plus surgery had survival rates only moderately below the expected values. However, for the group treated with radiation alone, survival rates were significantly below expected levels for at least 20 years. In all treatment groups and for both sites, excess mortality was partly due to late recurrences of uterine cancer. The continuing high mortality of women with corpus tumors treated only by radiation appears to be partly due to the selection of more favorable patients for surgery, but the data suggest the existence of other factors. Whatever the explanation may be, patients with endometrial cancers treated solely with radiation are subject to a substantially greater risk of late recurrence than patients treated surgically.—J. Nat. Cancer Inst. 27: 239–257, 1961.

SINCE 1956 the National Cancer Institute and The Connecticut State Department of Health have been cooperating in a study of uterine cancer in Connecticut, which is based primarily on records of the Connecticut Cancer Registry. The registry collects data on cancer of all sites from hospital records, tumor-clinic records, and death certificates throughout the State. The history and organization of the registry have been described by Griswold and Cutler (*1*), and the internal operation of the registry by Greenberg (*2*). Statistical tabulations from the registry are

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[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

now available for cancer of the various sites, including basic data on cancer of the uterine cervix and corpus (*3*), but only a limited amount of information can be accurately collected and processed in such a large-scale and continuing operation. The uterine-cancer study, of which this is the first report, is based on essentially the same case material as the earlier monograph (*3*) and a more recent paper by Cutler *et al.* (*4*), but includes information not available from the ordinary operations of the registry. The most important differences are that cases originally reported as "uterus, part unspecified" have been re-examined and, where possible, assigned to cervix or corpus; longer follow-up information is available; more data are given on treatment, diagnosis, cause of death, and other items; and classification of stage is based on pretreatment information rather than on that available at the time of the patient's discharge from the hospital.

Data for all cases of cancer in a defined community, rather than for cases seen at a single hospital or a group of hospitals, are particularly valuable for two reasons: 1) They can be related to the base population to provide incidence, prevalence, and mortality rates. 2) They describe the entire group of cancer patients in the population rather than the selected patients seen at a single treatment center. Most cancer patients are treated in their home communities by physicians not specializing in cancer therapy, and the experience of the selected cases referred to large cancer-treatment centers may be different from that of the cases treated in local hospitals.

For many years the most popular measures of success of cancer therapy have been related to the 5-year period following diagnosis and treatment of tumors. It has been a common clinical impression that in the first 5 years nearly all recurrences or metastases will have been found, and that any harmful effects of treatment will have become manifest. The data given here show that 5-year follow-up of patients with cancer of the uterine cervix or corpus is inadequate to evaluate the effects of the tumor and the results of treatment, and that there may be no single adequate period. These data thus add weight to the current concept that the *pattern* of death and survival rates is more informative than results after any specified period.

MATERIALS AND METHODS

Records routinely submitted to the Connecticut Cancer Registry were used to identify cases and supply basic data. Additional information was collected from hospital records, physicians' notes on follow-up, and death certificates. A methodological study comparing the results of routine registry operations with results of this special survey will be reported elsewhere; in general, agreement between the two methods was fairly good. The entire job of checking and abstracting data was done by a

single physician (JCB), which thus permitted uniformity in definitions and abstracting procedures.

The study covered hospitals with about 97 percent of all general hospital beds in the State. The remaining 3 percent were beds in small community hospitals known to refer cancer patients to other institutions for treatment. In addition, records were examined at 2 out-of-State hospitals known to treat Connecticut residents. In each hospital visited, the hospital and tumor-clinic files were searched for records of patients with uterine cancer who had not been reported to the central registry, which thus permitted a check on the completeness of reporting. The study is thought to include nearly all the diagnosed cases of uterine cancer and close to 100 percent of the treated cases.

This report pertains to 5,567 Connecticut residents with malignant neoplasms of the uterus seen in Connecticut hospitals, who were diagnosed from 1935 through 1951. An additional 854 cases were identified only by reports of uterine cancer on death certificates. Presumably, these women were not admitted to Connecticut hospitals for diagnosis or treatment of uterine cancer.

Of all patients seen in hospitals, 7.7 percent did not have microscopic confirmation of the diagnosis. However, 5-year survival without microscopic proof of cancer was low (21% for cervix and 15% for corpus), so that these patients have little influence on statistics of survival after the 5th year. The small number of benign neoplasms and other diseases that may be included in the group without pathologic confirmation of the diagnosis is probably less important than the larger number of malignant diseases that would be omitted if this group were ignored. In critical situations figures have been prepared for the microscopically confirmed cases only. In every instance they show essentially the same picture as that for all cases combined.

Of the total number of patients studied, 3,173 had tumors primary in the cervix and 2,191 had tumors primary in the corpus (table 1). In 203 cases, mostly reported only as "cancer of the uterus," it was impossible to tell from the records alone whether cervix or corpus was the primary site, but a study of reporting practices, internal statistical evidence, and other factors indicates that when Connecticut physicians say "cancer of the uterus" they nearly always mean "cancer of the uterine corpus." This is reflected in the histologic types of these cancers (table 1) and in a comparison of the site originally reported by the hospitals with the site as determined on review of the records. Of 963 tumors originally unspecified as to site, 717 were reclassified as corpus, 75 were reclassified as cervix, and 171 remain unspecified. Therefore, the unspecified group has been included with corpus in the following tables. It is thought that the relatively small number of cervical cancers misclassified by this practice will cause less error than the larger number of corpus cancers that would be excluded if the unspecified group were ignored. Carcinomas *in situ* and chorionic tumors are included. These were numerically unimportant in the computation of survival rates,

especially after the 8th year, since in most cases either the diagnoses were relatively recent (carcinomas *in situ*) or the patients did not survive very long (chorionic tumor).

TABLE 1.—Histologic types of uterine cancer, by site—Connecticut, 1935–51

Type and source	Total uterus	Cervix	Corpus	Not specified
All malignant tumors reported by hospitals	5, 567	3, 173	2, 191	203
Squamous carcinomas	2, 289	2, 253	28	8
Adenocarcinomas	2, 168	278	1, 805	85
Carcinomas, not further specified	250	214	29	7
Sarcomas	226	21	190	15
Carcinomas *in situ*	176	165	11	0
Chorionic tumors	25	0	22	3
Other and unspecified malignant tumors	7	3	4	0
Diagnosis not microscopically confirmed	426	239	102	85

SURVIVAL

Cases diagnosed during the 17-year period 1935 to 1951 were followed through 1956. The disposition of these cases by individual years of follow-up is shown in table 2. All had a potential follow-up of at least 5 years. In the table, "withdrawn alive" refers to persons who could not be followed further because the study closed during that year of observation. "Effective numbers exposed to risk" were calculated on the assumption that patients lost or withdrawn alive were followed for an average of 6 months of the year. Columns (6), (7), and (8) show the observed survival rates for patients year by year after diagnosis of uterine cancer, annual survival rates for the general population of females in Connecticut with the same age distribution at the beginning of each year of observation and followed during the same calendar period, and the ratios of these two rates. Ratios of observed (cancer population) to expected (general population) survival rates, called relative survival rates, permit the comparison of survival in different groups despite differences in age distribution, time of occurrence, or differential "normal" mortality within groups. Thus a relative survival rate of 1.00 means that survival in the group studied is the same as survival in the corresponding general population, while a rate above or below 1.00 means that observed survival is respectively higher or lower than expected survival, whatever the nature of the population studied.

The rates given in column (8) are for single years and must be multiplied in series to get relative survival rates for longer periods. This has been done in column (9) to give relative survival rates for the entire period since diagnosis. To find relative survival rates for other intervals, such as the 5- to 10-year period, one would multiply the annual rates for the appropriate years only. Cutler *et al.* (*5*) have given a more detailed

TABLE 2.—Annual survival rates 0 to 20 years after diagnosis of cancer of the uterus

Year of follow-up	Alive at beginning of year	Died during year	Lost during year	Withdrawn alive during year*	Effective numbers exposed to risk	Observed annual survival rate	Expected annual survival rate	Annual relative survival rate	Cumulative relative survival rate
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
A. Uterine cervix									
0–1	3,173	789	33		3,156.5	0.750	0.987	0.760	0.760
1–2	2,351	525	19		2,341.5	.776	.987	.786	.597
2–3	1,807	224	17		1,798.5	.875	.986	.887	.530
3–4	1,566	123	29		1,551.5	.921	.986	.934	.495
4–5	1,414	75	31		1,398.5	.946	.986	.959	.475
5–6	1,308	66	58	90	1,234.0	.947	.985	.961	.456
6–7	1,094	61	43	90	1,027.5	.941	.985	.955	.436
7–8	900	31	32	76	846.0	.963	.984	.980	.427
8–9	761	26	25	78	709.5	.963	.983	.980	.418
9–10	632	26	23	62	589.5	.956	.982	.974	.407
10–11	521	22	28	54	480.0	.954	.981	.972	.396
11–12	417	30	17	43	387.0	.922	.981	.940	.372
12–13	327	7	17	33	302.0	.977	.980	.997	.371
13–14	270	10	11	24	252 5	.960	.979	.981	.364
14–15	225	10	13	24	206.5	.952	.977	.974	.355
15–16	178	5	11	25	160.0	.969	.974	.995	.353
16–17	137	10	7	25	121.0	.917	.972	.943	.333
17–18	95	8	3	21	83.0	.904	.972	.930	.309
18–19	63	3	3	9	57.0	.947	.970	.976	.302
19–20	48	4	5	11	40.0	.900	.963	.935	.282
B. Uterine corpus, and uterus not specified cervix or corpus									
0–1	2,394	506	32		2,378.0	.787	.981	.802	.802
1–2	1,856	220	17		1,847.5	.881	.980	.899	.721
2–3	1,619	175	21		1,608.5	.891	.980	.909	.655
3–4	1,423	112	28		1,409.0	.921	.980	.940	.616
4–5	1,283	63	29		1,268.5	.950	.980	.969	.597
5–6	1,191	57	30	101	1,125.5	.949	.979	.969	.578
6–7	1,003	34	18	102	943.0	.964	.978	.985	.570
7–8	849	40	20	90	794.0	.950	.977	.972	.554
8–9	699	35	20	75	651.5	.946	.976	.969	.537
9–10	569	21	25	59	527.0	.960	.975	.985	.529
10–11	464	15	22	48	429.0	.965	.974	.991	.524
11–12	379	12	21	30	353.5	.966	.973	.993	.520
12–13	316	21	8	37	293.5	.928	.971	.956	.497
13–14	250	10	15	22	231.5	.957	.970	.987	.491
14–15	203	15	9	18	189.5	.921	.966	.953	.468
15–16	161	8	8	37	138.5	.942	.965	.976	.457
16–17	108	7	6	15	97.5	.928	.966	.961	.439
17–18	80	4	6	14	70.0	.943	.965	.977	.429
18–19	56	3	4	13	47.5	.937	.961	.975	.418
19–20	36	2	2	11	29.5	.932	.957	.974	.407

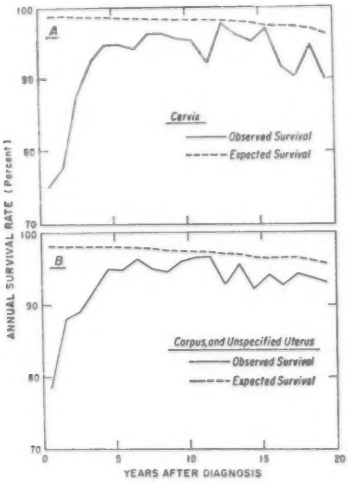
*Patients who could not be followed longer because the study closed during this year of observation

discussion of the methods used in computing and using relative survival rates. The value of this method for calculation of survival rates and the rationale for comparison of survival of cancer patients with survival in the general population have been described by others (*6–8*).

The validity of the relative survival rates depends on whether the expected rates are appropriate. The incidence of cancer of many sites, including the uterine cervix and corpus, is related to such factors as

income, marital status, and place of residence, and these variables are associated with general mortality rates. Unfortunately we do not have good data on the degree of association. For example, until life tables are available for various social and economic groups living in the same area, expected mortality rates must be based on all social and economic groups combined. However, Cutler (*9*) has shown that if the annual *death* rates of the standard population are within as wide a range as 50 percent of the true "normal" death rates for the population studied, the bias in annual relative survival rates is small. At present we can only say that expected mortality from causes other than cancer is probably about the same for cancer-susceptible individuals as for the rest of the population. Since the nonwhite Connecticut population was small in the years studied (2.2%), our expected mortality rates are based on the 1940 and 1950 life tables for the white population of the State, with interpolation or extrapolation to the midpoint of each follow-up year, and with adjustment to the age-distribution of the cancer population alive at the beginning of that year.

The annual observed and expected survival rates of table 2 are plotted in text-figure 1. For cancer of both cervix and corpus, survival rates in the first few years were low, but they rapidly increased, and, after about 5 years, survival rates for those still living were fairly close to the expected rates.



TEXT-FIGURE 1.—Observed and expected annual survival rates of uterine cancer patients. Irregularities in the two curves of expected survival are due to the shifting age distributions of survivors.

The improvement did not continue, and annual survival rates from the 5th year to the end of the observation period remained at about 97 percent of the expected value. The excess mortality of these cases over a

long period has been noted (*4*). The differences between observed and expected survival are almost the same for cervix and corpus, two sites closely related anatomically but different in most other respects. Annual survival rates for patients with cancer of the breast also remain at about 97 percent of the expected rates for many years after diagnosis (*5*).

One may ask if the low relative survival rates, or high mortality rates, are statistical artifacts due to loss of some patients to follow-up. Although 766 (13.8%) of patients are labeled "lost" during the period of 20 years (table 2), 5-year follow-up was 95.4 percent complete. The completeness of follow-up was about the same for the two sites. The high proportion of patients lost is partly due to the fact that the registry must rely on follow-up data voluntarily submitted by many hospitals and partly due to the definition of lost as "not known to be alive on the closing date of the study (December 31, 1956)." Choice of an earlier closing date would have substantially decreased the proportion classified as lost. A separate study of reporting practices and ways in which patients are "lost" and "found again" showed that most of the "lost" patients are under regular supervision by their physicians, but that follow-up reporting after 5 years is lax. Since reporting of deaths seems to be prompt and fairly complete, patients temporarily "lost" should have little effect on survival calculations based on life-table methods. This conclusion is supported by the results of intensive follow-up of a series of patients with breast cancer (*10*). Although 5.0 percent of the breast-cancer series was originally reported as lost, long-term survival rates based on life-table methods were very close to survival rates based on 99.8 percent complete follow-up of the same group. The true survival rates for uterine-cancer patients may have been slightly higher than the computed rates given here; however, even if *all* lost patients were still alive on December 31, 1956, the annual relative survival rates would be increased an average of less than 0.75 percentage points in each of the first 15 years, and an average of less than 1.2 percentage points in each of the first 20 years. Assuming that an appreciable proportion of lost patients are dead would decrease survival rates. The survival rates reported here are therefore close to the maximum possible values in spite of the number of patients not under current follow-up.

EFFECT OF AGE AND STAGE

Table 3 shows the age and stage distribution at the time of diagnosis for the entire group of cases studied. The definitions of stage used are:

in situ: intraepithelial carcinoma;
localized: confined to the site of origin (cervix, corpus, or entire uterus, for the three sites, respectively);
regional: extension beyond the site of origin but not beyond the true pelvis, and not involving the mucosa of the rectum or bladder;
distant: extension beyond the true pelvis, or involvement of the mucosa of the rectum or bladder, or both;
stage unknown: recorded pretreatment information insufficient to determine stage.

The stage given was based on information obtained prior to treatment. In cases of doubt, the earlier stage was recorded. These classifications are similar to the League of Nations' definitions for stage of cervical cancers, with pooling of League of Nations' stages III and IV as "distant," but our classification is based on recorded information rather than direct examination of patients.

TABLE 3.—Age and stage distribution of patients

	All ages	Age at diagnosis: Under 35	35–44	45–54	55–64	65–74	75 and over	Median age
Cervix								
All stages	*3, 173*	*226*	*758*	*906*	*779*	*387*	*117*	*52. 2*
In situ	165	33	68	43	13	7	1	42. 8
Localized	1, 488	113	388	463	332	154	38	50. 7
Regional	1, 232	68	255	337	353	167	52	54. 2
Distant	218	10	26	49	65	47	21	59. 2
Stage unknown	70	2	21	14	16	12	5	54. 1
Corpus and unspecified								
All stages	*2, 394*	*59*	*212*	*634*	*790*	*528*	*171*	*59. 2*
In situ	11	2	1	4	4	0	0	51. 8
Localized	1, 833	49	175	507	603	371	128	58. 6
Regional	308	3	21	80	105	84	15	60. 3
Distant	171	5	11	30	52	54	19	63. 1
Stage unknown	71	0	4	13	26	19	9	62. 6

Table 4 shows relative survival rates for 5-year periods in the first 20 years after diagnosis, for various age and stage groups. Both factors are strongly related to choice of treatment and to relative survival rates in the first 5 years, but their later effects on survival are much smaller. Relative survival rates for single years, not given here, show that the association between the clinical stage of uterine cancer and observed relative survival

TABLE 4.—Relative survival rates 0 to 5, 5 to 10, 10 to 15, and 15 to 20 years after diagnosis of cancer, by age and by stage*

	Cervix				Corpus and unspecified			
	0–5 years	5–10 years	10–15 years	15–20 years	0–5 years	5–10 years	10–15 years	15–20 years
Age:								
<35	0. 55	0. 91	0. 98	1. 02	0. 74†	0. 94	1. 01	1. 02
35–44	. 55	. 93	. 92	. 82†	. 80	. 96	. 95	. 89††
45–54	. 52	. 87	. 92	. 92†	. 74	. 91	. 93	. 86††
55–64	. 44	. 75	. 74†	‡	. 62	. 90	. 82†	. 92††
65–74	. 34	. 88†	‡	‡	. 41	. 79†	‡	—
75+	. 29†	‡	‡	—	. 36†	‡	‡	—
All ages	. 48	. 86	. 87	. 80†	. 60	. 89	. 88	. 88†
Stage:								
In situ	1. 00	. 91†	‡	—	. 96†	—	—	—
Localized	. 64	. 87	. 93	. 88†	. 71	. 90	. 88	. 92†
Regional	. 30	. 79	. 81†	. 62††	. 33	. 75†	. 87††	‡
Distant	. 05	. 96††	‡	—	. 03	‡	‡	—
Stage unknown	. 33†	. 87††	‡	‡	. 28†	‡	‡	††
All stages	. 48	. 86	. 87	. 80†	. 60	. 89	. 88	. 88†

*Stage based on pretreatment information only; standard error: no symbol = .00 to .049; † = .05 to .099; †† = .10 to .149; ‡ = .15 or greater; — = no cases observed through time interval.

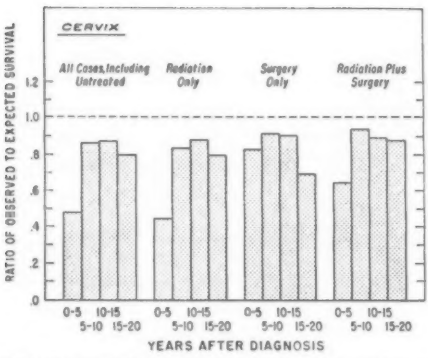
rates is small after the first 3 years following diagnosis. The effects of age and stage are considered small enough to permit pooling the data after 5 years. Since most of the 5-year survivors are drawn from the group of patients with tumors originally classified as localized (966 of 1,308 cervical tumors; 1,088 of 1,191 corpus tumors), long-term survival rates for all stages combined are very close to those for localized cases only.

EFFECT OF TREATMENT

The only variable studied that showed a strong association with survival rates after the 5th year was treatment. Relationships between treatment and survival in the first 5 years will be discussed more fully in a later paper, but it should be noted that the over-all Connecticut survival experience for both cervix and corpus compares favorably with that reported by leading treatment centers elsewhere in the United States (*4*). Excluding carcinomas *in situ*, total 5-year survival rates without correction for natural mortality were 44 percent for cervix and 59 percent for corpus.

There has been some discussion in the literature of the effect of good versus indifferent treatment. However, a discussion of the further survival experience of 5-year survivors is limited, by definition, to patients whose treatment was at least good enough to permit 5-year survival. It is worth remembering also that any study of long-term effects of treatment is necessarily based on treatment given many years earlier, and conclusions may not apply to current therapeutic practices. Patients treated with surgery are in general younger than those treated with radiation only, but the use of *relative* survival rates should reduce any age differences due to noncancer mortality, so that a direct comparison of treatment methods is possible. Since survival beyond 5 years did not seem to vary significantly with age or stage, the following discussion on the relations between survival and treatment is based primarily on all ages and stages combined. As shown in the tables, the combined figures are essentially those for patients with localized tumors only. Although most of the figures in this report are based on all uterine cancers reported, the picture is not materially changed if one considers only squamous carcinomas of the cervix and adenocarcinomas of the corpus.

Text-figure 2 shows relative survival rates in 5-year intervals for cervical cancers treated by surgery and radiation separately and in combination. The upper half of table 5 gives the number of cases in each treatment group with relative survival rates for all cases combined and for major histologic types. Survival in the 0- to 5-year period was strongly related to treatment. Survival rates between the 5th and 15th years were below the expected values in all treatment groups, and there were no significant differences between the 3 groups studied. Because of the small number of patients followed more than 15 years, the differences observed among survival rates after that point are not considered significant.



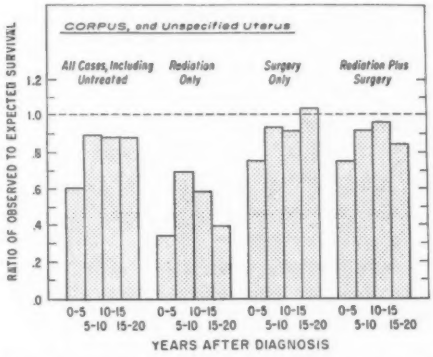
TEXT-FIGURE 2.—Relative survival rates of patients with cancer of the uterine cervix, by treatment group, for 20 years after diagnosis.

TABLE 5.—Relative survival rates 0 to 20 years after diagnosis for uterine cancer patients, by histologic type and treatment*

Cervix	Radiation only	Surgery only	Surgery and radiation
Number of cases	2, 348	363	248
Survival rates			
All cases:			
0–5 years	. 442	. 826	. 640
5–10 years	. 835	. 904	. 932
10–15 years	. 874	. 898	. 885
15–20 years	. 790	†	. 872
Squamous carcinomas:			
0–5 years	. 461	. 708	. 637
5–10 years	. 860	. 900	. 923
10–15 years	. 892	. 933	. 827
15–20 years	. 769	†	†
Adenocarcinomas:			
0–5 years	. 462	. 781	. 663
5–10 years	. 775	. 837	1. 090
10–15 years	. 740	†	†
15–20 years	†	†	†
Corpus			
Number of cases	538	944	702
Survival rates			
All cases:			
0–5 years	. 343	. 749	. 750
5–10 years	. 692	. 931	. 916
10–15 years	. 585	. 915	. 963
15–20 years	†	1. 034	. 842
Papillary adenocarcinomas:			
0–5 years	. 374	. 769	. 756
5–10 years	. 758	. 914	. 781
10–15 years	†	. 951	†
15–20 years	†	†	†
Adenocarcinomas, excluding papillary:			
0–5 years	. 410	. 806	. 812
5–10 years	. 749	. 938	. 947
10–15 years	. 616	. 919	. 967
15–20 years	†	1. 038	. 901
Sarcomas:			
0–5 years	†	. 518	†
5–10 years	†	. 953	†
10–15 years	†	. 957	†
15–20 years	†	†	†

*Untreated patients and patients with unknown treatment are omitted from this table.
†Fewer than 20 cases available for observation.

Text-figure 3 shows relative survival rates in 5-year intervals for cancer of the corpus, plus the unspecified group, treated by surgery and radiation separately and in combination. The lower half of table 5 gives relative survival rates for all reported patients and for the major histologic-type groups. Except for surgically treated patients after 15 years, survival was below the expected values in each time interval, but the excess mortality was consistently greater for patients treated by radiation only than for the others. Survival rates for radiologically treated patients reached a maximum between the 5th and 10th year, but showed a steady decrease in the second decade after diagnosis. However, the group treated only by radiation included many patients who could not be treated by surgery for one of two reasons: They were in poor general health, or their tumors had spread widely. The importance of this selective effect is discussed in a later selection. It may also be suggested that the excessive late mortality of radiation-treated corpus-cancer patients is due to deaths delayed until after the 5th year, and that these delayed deaths make radiation treatment seem less efficacious in the later period. This hypothesis cannot be examined directly, but it is not supported by the very low survival rates in the 0- to 5-year period.



TEXT-FIGURE 3.—Relative survival rates of patients with cancer of the uterine corpus, by treatment group, for 20 years after diagnosis.

Patients in the "radiation only" group have been subdivided into those receiving radium or radon, X radiation, or both. Relative survival rates for 0 to 5, 5 to 10, and 10 to 15 years are given in table 6. For cervix, survival after 5 years for patients receiving both forms of radiation was somewhat better than that of the group receiving radium or radon only, and considerably better than that of the group receiving only X radiation. For corpus the X radiation-only group is too small for analysis, but differences between the other two groups are not striking. Their survival remains very low, and may even decrease after the 10th year. More detailed study of these cases would be desirable, but death rates are so high that there are not enough survivors to permit analysis by age, stage of cancer, amount of radiation received, or other variables.

TABLE 6.—Relative survival rates of patients treated only by radiation*

Cervix	Number of patients	Relative survival rates 0–5 years	5–10 years	10–15 years
Total treated by radiation only	*2, 348*	*.44*	*.83*	*.87*
Radium or radon only	348	.51	.79	.78†
Radium or radon plus X radiation	1, 567	.50	.87	.92
X radiation only	433	.16	.57†	.59††
Corpus				
Total treated by radiation only	*538*	*.34*	*.69†*	*.59†*
Radium or radon only	209	.47	.76†	.57††
Radium or radon plus X radiation	189	.34	.64†	.41††
X radiation only	140	.16	.63††	1.08‡

*No symbol = standard error .00 to .049; † = .05 to .099; †† = .10 or greater; ‡ = SE indeterminate. No deaths observed among 6 patients.

COMPARISON OF OBSERVED AND EXPECTED MORTALITY

Since the relative survival rates presented here indicate that mortality remained at an above-normal level for 20 years after diagnosis, it is of interest to examine mortality *per se*. The data at hand show which of the patients were thought to have had uterine cancer at the time of death. For both cervix and corpus, the term "with uterine cancer" as used here applies to any patient dying with evidence of uterine malignancy at least 5 years after the original diagnosis, whether she was ever thought to be free of tumor, and whether the malignancy was in the same part of the uterus (cervix or corpus) as the original tumor. Metastases are included. Thus no distinction is made between true recurrences, recrudescences, and new primary tumors of the uterus. The difficulties of determining cause of death, and whether or not cancer is present at the time of death, are well known. A review of more than 200 recent deaths among 5-year survivors revealed that only 46 percent of the uterine malignancies reported as present at death had been microscopically confirmed. The proportion was about the same for patients originally diagnosed with cancers of the cervix and corpus. In spite of their limitations, examination of the available data seems worth while.

Table 7 shows, for each site, the number of deaths expected from general mortality rates and the actual number observed, by treatment and time interval after diagnosis. The observed numbers of deaths were classified as to the presence or absence of uterine cancer. Ratios of observed-to-expected numbers of deaths are also shown.

Among patients treated for cervical cancer, the observed total number of deaths in each treatment-time interval category was at least twice the expected number. For each type of treatment, the number of deaths without uterine cancer observed 5 to 20 years after diagnosis was somewhat greater (8 to 46%) than the number expected on the basis of general

TABLE 7.—Numbers of deaths in 5-year intervals, by treatment method and presence or absence of uterine cancer at death

Treatment method and follow-up interval	Expected deaths	Number of deaths observed			Ratios of observed deaths to expected deaths from all causes		
		Total	Without uterine cancer	With uterine cancer	Total	Without uterine cancer	With uterine cancer
Cervix							
Radiation only:							
5–10 years	63.4	174	68	106	2.74	1.07	1.67
10–15 years	28.8	61	37	24	2.12	1.28	0.83
15–20 years	11.3	25	20	5	2.21	1.77	0.44
Total 5–20 years	103.5	260	125	135	2.51	1.21	1.30
Surgery only:							
5–10 years	6.9	18	9	9	2.61	1.30	1.30
10–15 years	2.4	5	4	1	*	*	*
15–20 years	1.0	3	2	1	*	*	*
Total 5–20 years	10.3	26	15	11	2.52	1.46	1.07
Radiation and surgery:							
5–10 years	6.6	14	4	10	2.12	0.61	1.52
10–15 years	3.5	8	6	2	*	*	*
15–20 years	1.0	2	2	0	*	*	*
Total 5–20 years	11.1	24	12	12	2.16	1.08	1.08
Corpus and unspecified uterus							
Radiation only:							
5–10 years	19.2	54	27	27	2.81	1.41	1.41
10–15 years	6.8	21	12	9	3.09	1.76	1.32
15–20 years	1.5	5	4	1	*	*	*
Total 5–20 years	27.5	80	43	37	2.91	1.56	1.35
Surgery only:							
5–10 years	39.7	65	35	30	1.64	0.88	0.76
10–15 years	15.5	26	22	4	1.68	1.42	0.26
15–20 years	7.4	6	5	1	0.81	0.68	0.14
Total 5–20 years	62.6	97	62	35	1.55	0.99	0.56
Radiation and surgery:							
5–10 years	35.6	59	31	28	1.66	0.87	0.79
10–15 years	18.3	22	18	4	1.20	0.98	0.22
15–20 years	7.8	13	13	0	1.67	1.67	0
Total 5–20 years	61.7	94	62	32	1.52	1.00	0.52

*Ratios not computed when expected number of deaths was less than 5.

population experience. However, the excess mortality observed in each treatment group was mostly accounted for by deaths associated with uterine cancer.

Because of the relatively low proportion of patients with pathologic examination of late recurrences, no statistical analysis by histologic type is possible. However, available records give the impression that when late recurrences of squamous carcinomas of the cervix are reported, the recurrences are often reported as adenocarcinomas. This phenomenon could be due to the recurrence of glandular elements in tumors that were

at first predominately squamous, to the occurrence of new primary tumors in the cervix, or to other factors. It is interesting, also, that cervical cancers *originally* reported as adenocarcinomas seem to show late recurrences more often than those originally reported as squamous carcinomas. This is reflected in the relation between cell type and relative survival rates (table 5).

Among patients treated for cancer of the corpus, the total number of observed deaths exceeded the expected number in each treatment-time interval category except one (surgery only, 15–20 years). In contrast to the findings pertaining to cervical cancer, mortality 5 to 20 years after diagnosis varied by type of treatment. Total mortality was appreciably higher among patients treated by radiation only (mortality ratio of 2.91 compared with ratios of 1.55 and 1.52 for the other two treatment groups). Examination of the separate mortality ratios for deaths without uterine cancer reveals further differences. Among patients treated by radiation, the number of deaths without uterine cancer substantially exceeded the expected number. The ratio for the 5- to 20-year period of follow-up was 1.56. In contrast, in the two other treatment groups the number of deaths without uterine cancer was equal to the expected number. Furthermore, although deaths with uterine cancer contributed substantially to the observed excess mortality in all three treatment groups, such deaths occurred with greater relative frequency among patients treated by radiation only. The ratio of deaths associated with uterine cancer to the total number of expected deaths provides an arbitrary but useful means for comparison of the relative importance of such deaths among the three treatment groups. This ratio for corpus-cancer patients was 1.35 for the radiation treatment group and 0.56 and 0.52 for the two other groups.

Many patients with cancer of the uterine corpus are treated only with radiation because they fall into one of two categories: those called "inoperable" and those called "technically operable"; that is, those for whom operation is not indicated because of the extent of the cancer, and those for whom operation would be dangerous because of diseases other than cancer. The effect of selection of radiation treatment for patients in the first category, those with more advanced tumors, can be greatly reduced or eliminated by the restriction of comparisons to patients with localized cancers. Table 8 gives relative survival rates by treatment and time interval for two groups of patients: those with localized squamous-cell carcinomas of the cervix, and those with localized adenocarcinomas of the corpus. After the 0- to 5-year period, these figures are in substantial agreement with the data for all stages combined (table 5). Selection of corpus-cancer patients with advanced tumors for radiation treatment does not seem to explain the persistent high mortality of this group.

Many corpus-cancer patients were treated only by radiation because they were in poor general health, so it is not surprising that this group has somewhat higher death rates than the other treatment groups. Unfortunately, most of the medical records reviewed were incomplete with

TABLE 8.—Relative survival rates for patients with localized uterine cancers, for major histologic types and treatment groups*

	Radiation only	Surgery only	Radiation and surgery
Cervix—localized squamous cell-carcinomas			
Number of cases	839	125	126
Relative survival rates			
0–5 years	.63	.82	.73
5–10 years	.89	.89	.96
10–15 years	.94	.92	.81
15–20 years	.87	†	†
Corpus—localized adenocarcinomas‡			
Number of cases	245	555	462
Relative survival rates			
0–5 years	.50	.86	.85
5–10 years	.79	.95	.96
10–15 years	.54	.91	.95
15–20 years	†	1.01	.98

*Untreated patients and patients with unknown treatment are omitted from this table. Data based on microscopically proved tumors only.

†Fewer than 20 cases available for observation.

‡Excluding adenocarcinomas specified as papillary.

respect to diseases coexisting with cancer, and it was unusual to find any reasons given for the choice of one form of treatment rather than another. Therefore direct analysis of the effect of medical selection is not possible, and it is necessary to refer to data from other sources to estimate the order of magnitude of survival differences which could be attributed to selection of patients. We are not concerned here with the *immediate* effects of very serious illnesses, but rather with possible effects of poor health on the subsequent survival of patients alive 5 years after the diagnosis of cancer.

The limited data available suggest that mortality of persons originally in poor health remains high for many years. For example, this is true for persons with glycosuria, hypertension, or obesity. These three conditions are of particular interest because they have been linked with endometrial cancer and would be likely to cause selection of radiation rather than surgery for corpus-cancer patients. As shown in table 9, long-term mortality rates of several groups of persons with these conditions were between 114 and 164 percent of the expected mortality rates. These may be compared with the data on deaths without uterine cancer in table 7. Mortality for patients with endometrial cancer treated by radiation only was 156 percent of the number expected, *i.e.*, within the range observed among persons with glycosuria, hypertension, or obesity. Thus the excess deaths without uterine cancer might be attributed to selection on the basis of general health at the time of original treatment for cancer.

In addition to the excess deaths which may be attributed to medical selection, there were relatively more deaths with uterine cancer in the radiation-only group as compared to the two other treatment groups. There may be several explanations for the presence of malignant tumors in the uterus, years after the original diagnosis of cancer. First,

TABLE 9.—Long-term mortality rates of persons with obesity, hypertension, or glycosuria

Conditions	Individuals included	Sex	Time interval after observation (years)	Ratio of observed to expected mortality (percent)	Source
Obesity	Insured persons	Females	5–25	146	Dublin and Marks (*11*)
Essential hypertension	Clinic patients	Males and females	10–12	164	Palmer and Muench (*12*)*
Glycosuria	Insured persons:				
	Standard rates	Males and females	5–15	114	Society of Actuaries (*13*)*
	Substandard rates	Males and females	5–15	135	Society of Actuaries (*13*)*

*Data recalculated and summarized from the original.

they may be true continuations or recurrences of the original tumor, which arise from cells that have been dormant or growing slowly for a long period. Another possibility is that they may be new primary tumors arising in the same location as a result of the same factors which caused the first tumor. A third possibility, in patients treated radiologically, is that they may be new primary tumors induced by the radiation. Unfortunately there is no way to distinguish between these cases in practice, and all three mechanisms and perhaps others may be responsible. Whatever the explanation may be, the available data suggest that the patient with endometrial cancer who is treated solely with radiation is subject to a substantially greater risk of late "recurrence" than the patient treated surgically.

Until there is more direct evidence concerning the effect of medical selection on long-term survival rates, it will not be possible to say whether differences in survival between various treatment groups are due to selection of patients or to the effects of the treatments themselves. In the meantime, observational studies such as the one reported here offer the best means to investigate these differences and to estimate the long-term hazards associated with various treatment methods. A controlled experiment, *i.e.*, a clinical trial, to evaluate and compare the survival of different treatment groups would clearly be superior to observational studies. However, a controlled experiment would require many years just to collect enough cases for study (in this series, 17 years' experience in an entire State seems barely adequate), plus a further interval of years for the accumulation of survival experience. This would be beyond the facilities and patience of most investigators. If the question of long-term treatment effects is of current interest, it must be investigated by means of observational studies. Another observational study of long-term treatment effects is now in progress in Norway, which should provide a large

series of corpus-cancer patients for whom radiation therapy was the usual form of treatment in favorable, as well as unfavorable, cases.

OTHER REPORTS OF LONG-TERM SURVIVAL

There have been many reported studies of the survival of uterine-cancer patients after the 5th year, but very few permit a direct comparison of observed and expected survival. The well-known Annual Reports (*14*) give survival data at intervals up to 10 years after diagnosis and treatment. Survival rates by treatment can be computed for the 5- to 10-year interval, but wide variations among the reporting hospitals in populations of patients and expected mortality rates do not permit one to compare pooled survival rates with confidence.

Cutler *et al.* (*4*) have studied survival of uterine-cancer patients in the Connecticut Tumor Registry, and there is considerable overlap between their series and that reported here. The two reports are in close agreement on the long-term excess in mortality of uterine-cancer patients, and on the relative differences between treatment groups. Cutler *et al.* found that, for both cervix and corpus, survival rates were below expected values for at least 15 years, that survival after 5 years did not depend on the original stage of the disease, and that for corpus cancer, radiation treatment was associated with a substantially increased risk of death between the 5th and 15th years. They also examined time trends in late relative survival rates; from 1935–44 to 1945–54 there was a slight increase in relative survival between the 5th and 10th year from 85 to 89 percent for cervix, but no change in the ratio for corpus, 88 percent.

Sørensen (*15*) has recently reported on 798 patients with cervical cancer treated by radium from 1922 through 1929. He found that late recurrences were responsible for 34 (25%) of 136 deaths among 201 5-year survivors, and that the observed number of deaths exceeded the expected number by approximately the number of deaths with cervical cancer. The incidence of recurrences decreased logarithmically with time and the probability of a late recurrence was not related to the original stage of the disease. Other series on follow-up of cervical-cancer patients have been summarized by Sørensen (*15*). In general, the published data give no clear indication of a relation between the incidence of late recurrence and original stage, age at diagnosis, or treatment for cancer of the cervix.

Long-term recurrence and mortality data for corpus tumors are limited. Finn (*16*) found that 5 of 74 five-year survivors developed recurrences by the 11th year. The patients were originally treated by surgery if possible. Finn suggests a recurrence rate of 1 percent per year after the 3d year. Webb *et al.* (*17*) found that 27 of 98 five-year survivors of adenocarcinoma of the endometrium died in the succeeding 5 years, but the number of recurrences was not reported. There were 16 deaths in 36 patients treated by radiation only (44%), and 11 deaths in 62 patients treated by surgery alone or in combination with radiation (18%). Sur-

vival after the first 5 years was better for patients with localized tumors than for those with more extensive tumors, but was not related to the degree of differentiation of the original tumor. Burger (*18*) found no late recurrences after corpus carcinoma treated 1913 to 1923, when surgery was commonly used. From 1923 to 1935, when radiation was the rule, late recurrences occurred in 11.26 percent of patients surviving 5 or more years. The number of recurrences seemed to decrease after 12 years, but the population alive and under follow-up was also decreasing; data are not given for recurrence *rates*. Burger also found that cervical-cancer patients developed late recurrences more often if they had been treated with radiation than if they had been treated with surgery. Blaikley, commenting on a paper by Kottmeier (*19*), has denied that late recurrences of corpus cancer are much more common after radiotherapy than after surgery, but Kottmeier's table IX, based on the Annual Reports (*14*), shows that death rates (from all causes) between the 5th and 10th years are 25 percent for patients at institutions favoring primary surgery, and 31 percent at institutions favoring primary radiotherapy. Koch (*20*) found 32 late recurrences (including 10 "new cancers" of the uterus) in a group of 278 five-year survivors followed for varying periods. Nearly all of his cases were originally treated by radiotherapy only.

Most other reports on long-term follow-up of patients with cancer of the uterine corpus are based on very small series which are difficult to interpret. There is no consistent pattern in the published data, but recurrences seem to be somewhat more common, and survival rates somewhat lower, for patients treated by radiation than for those treated by surgery.

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Studies on the Suppression of the Homograft Response With Folic Acid Antagonists [1]

STEWART R. HUMPHREYS, MICHAEL A. CHIRIGOS, KENNETH L. MILSTEAD,[2] NATHAN MANTEL, *and* ABRAHAM GOLDIN, *Laboratory of Chemical Pharmacology and Biometry Branch, National Cancer Institute,[3] Bethesda, Maryland*

SUMMARY

Leukemia L1210 and antifolic-resistant variants M46R, M66-3R, and C82R were transplanted into incompatible strains of mice. In each foreign strain the tumor usually grew and regressed in typical homograft response. Surviving mice were resistant to challenge with the sensitive or resistant variant. The homograft response evidenced with the resistant tumors could be suppressed by administration of antifolics. In such case, treatment resulted in progressive tumor growth and death. The extent of suppression of homograft response appeared to be related to drug dosage. However, the antifolics did not interfere as readily with the induction of host immunity. Also, once immunity was established, it was not readily suppressed by antifolics. Antifolic treatment, which abrogated the homograft response to resistant leukemia, failed to interfere with the ability of an inoculum of sensitive L1210 or spleen to make the mice immune to reinoculation. Survivors of resistant as well as sensitive L1210 were obtained with delayed administration of antagonist and were immune to reinoculation. Doses of antifolic too low to overcome the homograft response with resistant variants failed to interfere with the development of immunity. Sufficiently extensive treatment with antifolics may interfere to some extent with the ability of normal or sensitive leukemic spleen to immunize incompatible strains of mice. It may even interfere somewhat with already established immunity. Suppression of the homograft response may not occur if a more effective folic acid antagonist is used. Instead, inhibition of tumor growth may result. The results are discussed with reference to therapy with antifolics.—J. Nat. Cancer Inst. 27: 259-276, 1961.

IN PREVIOUS studies it was demonstrated that mice surviving systemic L1210 leukemia after extensive therapy with the halogenated derivatives of amethopterin (AM) were immune to reinoculation of the sensitive or resistant sublines of the leukemia (*1–3*). Resumption of administration of 3′,5′-dichloroamethopterin (DCM) to mice reinoculated with an antifolic-resistant subline of L1210 leukemia failed to suppress the established immunity (*4*). Also, treatment of the M46R-resistant variant of L1210 in (BALB/cAn $\times$ DBA/2J)F_1 mice previously inoculated with

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[2] Summer Employee Program. *Current address:* Georgetown University Medical School, Washington, D.C.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

the sensitive leukemic line resulted in increased response of the M46R-resistant variant to therapy (*5*). It was suggested that the augmented therapeutic response was attributable to the elicitation of an immune response in the host on treatment of the sensitive leukemia, which, in turn, contributed to the therapy of the resistant variant.

Our studies were conducted in compatible ("semi"-isologous) first generation (BALB/cAn × DBA/2J)F_1 male mice, in which the DBA parent is the strain of origin of L1210 leukemia (*6*). The observations seemed to conflict with reports that folic acid antagonists may interfere with the homograft reaction in homologous strains of mice (*7*, *8*). Uphoff demonstrated that AM can prevent the homograft reaction in X-irradiated mice receiving injections of homologous bone marrow (*7*). Potter and Haas have shown that the homograft response to a subline of the P288 lymphocytic neoplasm resistant to AM can be overcome by treatment with AM (*8*).

In the isologous host, folic acid antagonists appear to permit the induction of immunity, while in the homologous host, as evidenced by suppression of the homograft response, they seem to interfere with immunological phenomena. To what extent these observations may be fundamentally contradictory has not been clarified. Tumor homograft inhibition and enhancement have been demonstrated in a single tumor system in accordance with temporal relationships of administration of the homografts (*9*) and the amount of antiserum administered (*10*). Possible interrelationships of immune phenomena influenced by folic acid antagonists have received little attention. Because of the apparently contradictory nature of the mode of action of antifolics with respect to immune responses, additional studies were undertaken in homologous animals on the action of folic acid antagonists on the sensitive L1210 leukemia and several antifolic-resistant sublines of this leukemia.

In agreement with the results with the P288 lymphocytic neoplasm (*8*), the current results show that the homograft response to antifolic-resistant sublines of L1210 leukemia may be overcome by treatment with AM or DCM. However, the folic acid antagonists do not interfere as extensively with the induction of host immunity and, once immunity is induced, it is not readily suppressed by treatment with folic acid antagonists.

METHODS

The general methods have been previously described (*1*, *2*, *11*). The experiments were conducted in male BALB/cAn mice; (BALB/cAn × A/LN), which will hereafter be called CAF_1; C57BL/6; and (BALB/cAn × DBA/2J)F_1, which will hereafter be called CDBA. The tumors used were the sensitive L1210 leukemia (subline Z) and the antifolic-resistant variants M46R, M66-3R, and C82R. The sensitive line was carried in DBA/2J mice; the resistant sublines were carried in CDBA mice. M46R is resistant to AM and relatively cross-resistant to DCM (*12*). M66-3R

and C82R are resistant to DCM and AM (*1*, *13*). The leukemic cell suspensions were prepared from spleen mash, as indicated in the tables and administered in the volume of 0.1 ml/mouse. Where successive inoculums were administered the first was in the right and the second in the left inguinal area, each in 0.1 ml of saline. Serial dilutions of the experimental inoculums were used where indicated.

AM and DCM were prepared in 2 percent sodium bicarbonate and were injected subcutaneously in the axillary area at 0.01 ml/g of body weight.

The details of each experiment are indicated in the tables.

RESULTS

The results of the experiments are given in tables 1 to 7. Each experiment, as shown in the tables, covered two time periods, corresponding to the two times at which tumor inoculations were made. Mice received inoculations at either or both times, and observations were made in both periods.

Observations covered in the tables include: the successful tumor takes in the incompatible host; the maximum diameter attained by a tumor during the period and its day of attainment; persistence of tumors as evidenced by the two latest times required in each group for the tumor to regress; and the proportion of mice surviving the period, together with the median survival time of those dying. Mice surviving the first observation period were available for treatment and further observation in the second period.

The results of each experiment are placed in sections on the basis of phenomena observed.

Experiment 1. Amethopterin suppressed homograft response but failed to alter established immunity

In the first experiment, the sensitive leukemic line L1210 and the AM-resistant variant M46R were used in BALB/cAn mice and the animals were treated with AM (table 1).

TABLES

Definition of Headings

Tumor takes: Mice with a 6 mm diameter or larger palpation measurement on at least 1 palpation day during the period. In the compatible host a 6 mm palpation is ordinarily indicative of the existence of a tumor.

Maximum tumor diameter, average: This is the average of the largest palpation measurement for mice showing tumor take.

Day of maximum diameter, average: This is the average postinoculation day for mice showing takes, on which the largest palpation measurement was made. Where palpation measurements are equal, the earlier day is used.

Tumor duration: This is the first postinoculation day that the palpation measurement drops and remains below 6. The "greater than" symbol indicates presence of tumor at day of death or termination of period. Only the 2 longest durations in each group are shown. A single figure is shown where both durations are equal or when there is only a single tumor take in the group.

Survival: This is shown as of end of the period. The median day of death, postinoculation day for the period, refers only to mice dying during the period; where only 1 mouse in a group dies, it is the day of death for that animal.

Section I.—The tumors grew locally at the site of inoculation when AM was not administered. Some animals died, but complete regression occurred in most cases.

Section II.—When the sensitive L1210 leukemia was inoculated into the BALB/cAn mice and they were treated with AM, there was complete inhibition of growth of local tumor.

Section III.—However, when the resistant variant M46R was inoculated and AM administered, the tumor grew progressively, and all but 1 animal died. In this experiment, the treated animals bearing M46R died at approximately the time the homograft response or death occurred in the untreated animals bearing M46R or L1210. In such a situation it is often difficult to determine to what extent the animals may have succumbed to progressive growth of the tumor, and to what extent the toxicity of the drug may have contributed to death. However, in this and other experiments, treated animals bearing antifolic-resistant leukemia died after receiving relatively nontoxic doses of drugs. In experiment 4 tumor regression and death were clearly delayed on treatment with AM or DCM.

Section I and IV.—The mice immunized with L1210 were immune to reinoculation of L1210 and M46R. Similarly, after growth and regression of the M46R variant, the mice were immune to reinoculation of M46R.

Section I and V.—After growth and regression of the sensitive L1210 leukemia or the resistant variant, treatment of reinoculated mice with AM did not interfere with the established immunity.

TABLE 1.—Effect of treatment with AM on homograft response and on immune response in reinoculated mice—(sensitive L1210Z leukemia and the amethopterin-resistant subline M46R)*

Group	Treatment AM Dose (mg/kg)	Treatment AM Schedule (daily)	Inoculum† (day 0)	First period (days 0–20) Tumor occurrence Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Survivors Median day of death	Inoculum‡ (day 21)	Second period (days 21–41) Tumor occurrence Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Survivors Median day of death
							I. Tumor growth and regression in incompatible host									
1	0		L1210Z	7/9	10.9	9.7	15	9/9							9/9	
2	0		M46R	9/9	12.3	9.4	17, 20	5/9	10						5/5	
3	0							9/9		L1210Z	9/9	10.8	8.4	15	6/9	9
4	0							9/9		M46R	9/9	11.4	9.4	20, >20	7/9	12.5
							II. Inhibition by drug of sensitive tumor in incompatible host									
5	1.0	0–41	L1210Z	0/9				9/9							9/9	
6	1.0	21–41						9/9		L1210Z	0/9				9/9	
							III. Lethality with drug treatment of resistant tumor in incompatible host									
7	1.0	0–20	M46R	9/9	11.8	8.4	>11	0/9	11						0/0	
8	1.0	21–41						9/9		M46R	9/9	11.3	10.7	>14, >16	1/9	12
							I. Tumor growth and regression and								*IV. Immunity of incompatible host to reinoculation*	
9			L1210Z	8/9	10.4	9.8	17	9/9		L1210	0/9				9/9	
10			L1210Z	7/9	11.7	9.1	15, 17	9/9		M46R	0/9				9/9	
11			M46R	9/9	13.0	8.9	15	6/9	9	M46R	0/6				6/6	
							I. Tumor growth and regression and								*V. Retained immunity with drug treatment*	
12	1.0	21–41	L1210Z	9/9	10.4	9.7	15	9/9		L1210Z	0/9				9/9	
13	1.0	21–41	L1210Z	9/9	10.1	8.7	15, 17	9/9		M46R	0/9				9/9	
14	1.0	21–41	M46R	9/9	13.3	11.0	17, >20	7/9	13	M46R	0/7				6/7§	4
							Nontumorous Control									
15	1.0	0–41						9/9							9/9	
16								5/5							5/5	

*BALB/cAn male mice.

†One spleen plus 9 ml N saline for both leukemias. The inoculum concentration for each leukemia was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice.

‡One spleen plus 9 ml N saline for both leukemias. The inoculum concentration for each leukemia was 1000 times greater than that required to give 100 percent takes in control CDBA hybrid male mice.

§This group inadvertently treated with AM on day 8. In 1 mouse the tumor eventually attained a diameter of 18 mm (in the second period) and the mouse died on day 25 (day 4 for the second period).

TABLE 2.—Effect of treatment with DCM on homograft response and on immune response in reinoculated mice—(sensitive L1210Z leukemia and the DCM-resistant subline C82R)*

Treatment DCM			First period (days 0–20)							Second period (days 21–42)						
				Tumor occurrence				Survivors			Tumor occurrence				Survivors	
					Maximum tumor diameter							Maximum tumor diameter				
Group	Dose (mg/kg)	Schedule daily	Inoculum† (day 0)	Takes Total	Average (mm)	Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death	Inoculum‡ (day 21)	Takes Total	Average (mm)	Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death
							I. Tumor growth and regression in the incompatible host									
1	0		L1210Z	8/8	12.1	8.8	20, >20	5/8	10						5/5	
2	0		C82R	8/8	11.0	12.1	20	8/8							8/8	
3	0							8/8		L1210Z	4/8	9.5	9.5	14, 17	8/8	
4	0							8/8		C82R	8/8	11.2	9.1	17, 20	6/8	10.5
							II. Inhibition by drug of the sensitive tumor in the incompatible host									
5	60	0–42	L1210Z	2/9	6.0	9.0	9, 13	9/9							7/9	7
6	75	0–42	L1210Z	1/9	6.0	8.0	>10	6/9	18						5/6	19
7	60	21–42						8/8		L1210Z	0/8				3/8§	16
8	75	21–42						8/8		L1210Z	0/8				5/8	18
							III. Lethality with drug treatment of the resistant tumor in the incompatible host									
9	60	0–42	C82R	9/9	10.6	11.2	17, >20	2/9‖	13						1/2	12
10	75	0–20	C82R	9/9	9.0	9.9	>14	0/9	13						0/0	
11	60	21–42						8/8		C82R	8/8	10.9	9.1	>13, >15	0/8	11
12	75	21–42						8/8		C82R	8/8	11.1	9.2	>12, >14	0/8	11
							I. Tumor growth and regression and *IV. Immunity of the incompatible host to reinoculation*									
13	0		L1210Z	9/9	12.1	8.6	>20	8/9	10	L1210Z	0/8				8/8	
14	0		L1210Z	9/9	12.4	8.4	>20	7/9	9	C82R	0/7				7/7	
15	0		C82R	9/9	11.3	10.6	20	8/9	12	C82R	0/8				8/8	
							I. Tumor growth and regression and *V. Retained immunity with drug treatment*									
16	60	21–42	L1210Z	9/9	12.9	8.7	17, 20	8/9	9	L1210Z	0/8				4/8	15
17	75	21–42	L1210Z	9/9	11.7	8.1	17, >20	8/9	10	L1210Z	0/8				5/8	16
18	60	21–42	L1210Z	9/9	13.3	8.7	>20	9/9		C82R	0/9				7/9	18
19	75	21–42	L1210Z	9/9	12.7	8.7	17, >20	8/9	9	C82R	0/8				7/8	18
20	60	21–42	C82R	9/9	10.3	11.4	20, >20	9/9		C82R	0/9				8/9	16
21	75	21–42	C82R	9/9	9.2	11.1	17	9/9		C82R	0/9				2/9	14

*BALB/cAn male mice.

†One spleen plus 9 ml N saline for both leukemias. Inoculum concentration for the L1210Z leukemia was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice. For the C82R variant the inoculum concentration was 10 times greater than that required to give 100 percent takes in these mice.

‡One spleen plus 9 ml N saline for both leukemias. Inoculum concentration for both leukemias was sufficient to give 100 percent takes in control CDBA hybrid male mice. No titration conducted.

§DCM was somewhat toxic in this experiment. A number of animals died with marked weight loss and no evidence of tumor growth.

‖One survivor had a large tumor, attaining a 15 mm diameter, from which the mouse died on day 33 (day 13 of the second period).

TABLE 3.—Effect of schedule of treatment with DCM on the homograft response and on the development of host immunity—sensitive L1210Z leukemia, the DCM-resistant subline C82R, and normal DBA/2J spleen mash*

conducted.

§DCM was somewhat toxic in this experiment. A number of animals died with marked weight loss and no evidence of tumor growth.

¶One survivor had a large tumor, attaining a 15 mm diameter, from which the mouse died on day 33 (day 12 of the second period).

TABLE 3.—*Effect of schedule of treatment with DCM on the homograft response and on the development of host immunity—sensitive L1210Z leukemia, the DCM-resistant subline C82R, and normal DBA/2J spleen mash**

Treatment DCM			First period (days 0–26)							Second period (days 27–50)						
				Tumor occurrence				Survivors			Tumor occurrence				Survivors	
Group	Dose (mg/kg)	Schedule (daily)	Inoculum† (day 0)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death	Inoculum‡ (day 27)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death
			I. Tumor growth and regression in the incompatible host													
1								12/12		C82R	12/12	11.6	7.8	16	4/12	9
			I. Tumor growth and regression					and		*IV. Immunity of incompatible host to reinoculation*						
2			L1210Z	8/10	6.8	7.0	15	10/10		C82R	0/10				10/10	
3			C82R	9/10	9.4	11.0	17	10/10		C82R	0/10				10/10	
			II. Inhibition by drug of sensitive tumor					and		*VI. Development of immunity despite drug treatment*						
4	60	0–26	L1210Z	0/10				10/10		C82R	2/10	7.5	7.0	9	10/10	
5	60	7–26	L1210Z	7/10	6.0	6.0	10	10/10		C82R	0/10				10/10	
			III. Lethality with drug treatment of the resistant tumor					and		*VI. Development of immunity despite drug treatment*						
6	60	0–26	C82R	10/10	9.7	11.8	>15	0/10	14						0/0	
7	60	7–26	C82R	9/10	10.7	13.3	24, 26	6/10	15	C82R	0/6				6/6	
			VII. Spleen induced immunity to tumor inoculation; drug interference with immunity either absent or partial													
8			Spleen					10/10		C82R	1/10	9.0	13.0	16	10/10	
9	60	0–26	Spleen					10/10		C82R	0/10				10/10	
10	60	7–26	Spleen					10/10		C82R	7/10	7.0	8.0	9, 11	10/10	

*BALB/cAn male mice.

†One spleen plus 5 ml N saline for L1210Z and C82R. Four spleens plus 5 ml N saline for normal DBA/2J spleen. Inoculum concentration for the L1210Z leukemia was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice. For the C82R variant the concentration was 10 times greater than that required to give 100 percent takes in these mice.

‡One spleen plus 10 ml N saline. Inoculum concentration was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice.

TABLE 2.—Effect of treatment with DCM on homograft response and on immune response in reinoculated mice—(sensitive L1210Z leukemia and the DCM-resistant subline C82R)*

Treatment DCM			First period (days 0–20)							Second period (days 21–42)						
				Tumor occurrence				Survivors			Tumor occurrence				Survivors	
					Maximum tumor diameter							Maximum tumor diameter				
Group	Dose (mg/kg)	Schedule daily	Inoculum† (day 0)	Takes Total	Average (mm)	Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death	Inoculum‡ (day 21)	Takes Total	Average (mm)	Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death
							I. Tumor growth and regression in the incompatible host									
1	0		L1210Z	8/8	12. 1	8. 8	20, >20	5/8	10						5/5	
2	0		C82R	8/8	11. 0	12. 1	20	8/8							8/8	
3	0							8/8		L1210Z	4/8	9. 5	9. 5	14, 17	8/8	
4	0							8/8		C82R	8/8	11. 2	9. 1	17, 20	6/8	10. 5
							II. Inhibition by drug of the sensitive tumor in the incompatible host									
5	60	0–42	L1210Z	2/9	6. 0	9. 0	9, 13	9/9							7/9	7
6	75	0–42	L1210Z	1/9	6. 0	8. 0	>10	6/9	18						5/6	19
7	60	21–42						8/8		L1210Z	0/8				3/8§	16
8	75	21–42						8/8		L1210Z	0/8				5/8	18
							III. Lethality with drug treatment of the resistant tumor in the incompatible host									
9	60	0–42	C82R	9/9	10. 6	11. 2	17, >20	2/9‖	13						1/2	12
10	75	0–20	C82R	9/9	9. 0	9. 9	>14	0/9	13						0/0	
11	60	21–42						8/8		C82R	8/8	10. 9	9. 1	>13, >15	0/8	11
12	75	21–42						8/8		C82R	8/8	11. 1	9. 2	>12, >14	0/8	11
							I. Tumor growth and regression and *IV. Immunity of the incompatible host to reinoculation*									
13	0		L1210Z	9/9	12. 1	8. 6	>20	8/9	10	L1210Z	0/8				8/8	
14	0		L1210Z	9/9	12. 4	8. 4	>20	7/9	9	C82R	0/7				7/7	
15	0		C82R	9/9	11. 3	10. 6	20	8/9	12	C82R	0/8				8/8	
							I. Tumor growth and regression and *V. Retained immunity with drug treatment*									
16	60	21–42	L1210Z	9/9	12. 9	8. 7	17, 20	8/9	9	L1210Z	0/8				4/8	15
17	75	21–42	L1210Z	9/9	11. 7	8. 1	17, >20	8/9	10	L1210Z	0/8				5/8	16
18	60	21–42	L1210Z	9/9	13. 3	8. 7	>20	9/9		C82R	0/9				7/9	18
19	75	21–42	L1210Z	9/9	12. 7	8. 7	17, >20	8/9	9	C82R	0/8				7/8	18
20	60	21–42	C82R	9/9	10. 3	11. 4	20, >20	9/9		C82R	0/9				8/9	16
21	75	21–42	C82R	9/9	9. 2	11. 1	17	9/9		C82R	0/9				2/9	14

*BALB/cAn male mice.

†One spleen plus 9 ml N saline for both leukemias. Inoculum concentration for the L1210Z leukemia was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice. For the C82R variant the inoculum concentration was 10 times greater than that required to give 100 percent takes in these mice.

‡One spleen plus 9 ml N saline for both leukemias. Inoculum concentration for both leukemias was sufficient to give 100 percent takes in control CDBA hybrid male mice. No titration conducted.

§DCM was somewhat toxic in this experiment. A number of animals died with marked weight loss and no evidence of tumor growth.

‖One survivor had a large tumor, attaining a 16 mm diameter, from which the mouse died on day 33 since 12 of the second period.

TABLE 3.—Effect of schedule of treatment with DCM on the homograft response and on the development of host immunity—sensitive L1210Z leukemia, the DCM-resistant subline C82R, and normal DBA/2J spleen mash*

conducted.

‖DCM was somewhat toxic in this experiment. A number of animals died with marked weight loss and no evidence of tumor growth.

§One survivor had a large tumor, attaining a 16 mm diameter, from which the mouse died on day 88 (day 42 of the second period).

TABLE 3.—Effect of schedule of treatment with DCM on the homograft response and on the development of host immunity—sensitive L1210Z leukemia, the DCM-resistant subline C82R, and normal DBA/2J spleen mash*

Treatment DCM			First period (days 0–26)							Second period (days 27–50)						
				Tumor occurrence				Survivors			Tumor occurrence				Survivors	
Group	Dose (mg/kg)	Schedule (daily)	Inoculum† (day 0)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death	Inoculum‡ (day 27)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death
			I. Tumor growth and regression in the incompatible host													
1								12/12		C82R	12/12	11.6	7.8	16	4/12	9
			I. Tumor growth and regression					and		*IV. Immunity of incompatible host to reinoculation*						
2			L1210Z	8/10	6.8	7.0	15	10/10		C82R	0/10				10/10	
3			C82R	9/10	9.4	11.0	17	10/10		C82R	0/10				10/10	
			II. Inhibition by drug of sensitive tumor					and		*VI. Development of immunity despite drug treatment*						
4	60	0–26	L1210Z	0/10				10/10		C82R	2/10	7.5	7.0	9	10/10	
5	60	7–26	L1210Z	7/10	6.0	6.0	10	10/10		C82R	0/10				10/10	
			III. Lethality with drug treatment of the resistant tumor					and		*VI. Development of immunity despite drug treatment*						
6	60	0–26	C82R	10/10	9.7	11.8	>15	0/10	14						0/0	
7	60	7–26	C82R	9/10	10.7	13.3	24, 26	6/10	15	C82R	0/6				6/6	
			VII. Spleen induced immunity to tumor inoculation; drug interference with immunity either absent or partial													
8			Spleen					10/10		C82R	1/10	9.0	13.0	16	10/10	
9	60	0–26	Spleen					10/10		C82R	0/10				10/10	
10	60	7–26	Spleen					10/10		C82R	7/10	7.0	8.0	9, 11	10/10	

*BALB/cAn male mice.

†One spleen plus 5 ml N saline for L1210Z and C82R. Four spleens plus 5 ml N saline for normal DBA/2J spleen. Inoculum concentration for the L1210Z leukemia was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice. For the C82R variant the concentration was 10 times greater than that required to give 100 percent takes in these mice.

‡One spleen plus 10 ml N saline. Inoculum concentration was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice.

TABLE 4.—Influence of dose level of AM or DCM on homograft response and on development of host immunity in mice inoculated with resistant variants of L1210 leukemia—amethopterin-resistant subline M46R and DCM-resistant subline M66-3R*

Treatment			First period (M46R days 0–35) (M66-3R days 0–34)							Second period (M46R days 36–59) (M66-3R days 35–58)						
				Tumor occurrence				Survivors			Tumor occurrence				Survivors	
Group	Dose (mg/kg)	Schedule daily	Inoculum† (day 0)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death	Inoculum‡ (day 35 or 36)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death
			I. Tumor growth and regression in the incompatible host													
1	0							5/5		M46R	5/5	10. 6	7. 6	19	5/5	
2	0							5/5		M66-3R	5/5	12. 2	7. 4	17	5/5	
			I. Tumor growth and regression					and		*IV. Immunity of the incompatible host to reinoculation*						
3	0		M46R	8/10	6. 1	6. 5	11	10/10		M46R	1/10	8. 0	8. 0	12	10/10	
4	0		M66-3R	7/10	6. 7	7. 0	10	10/10		M66-3R	0/10				10/10	
			III. Lethality with drug treatment of the resistant tumor					and		*V., VI. Development and retention of immunity despite drug treatment*						
	AM															
5	0. 25	−2–59	M46R	5/5	7. 6	7. 1	15	5/5		M46R	0/5				5/5	
6	0. 39	−2–59	M46R	5/5	7. 8	10. 4	18, 20	5/5		M46R	3/5	8. 3	13. 0	17, 19	5/5	
7	0. 61	−2–59	M46R	4/5	10. 8	16. 0	>22, >25	1/5	17	M46R	0/1				1/1	
8	0. 96	−2–35	M46R	5/5	10. 0	12. 2	>16, >17	0/5	15						0/0	
9	1. 50	−2–59	M46R	4/5	9. 5	12. 0	>14, >20	1/5	13	M46R	1/1	11. 0	10. 0	>11	0/1	11
	DCM															
10	25	−3–58	M66-3R	5/5	7. 8	6. 6	12, 17	5/5		M66-3R	0/5				5/5	
11	39	−3–58	M66-3R	5/5	8. 4	10. 2	18, 25	5/5		M66-3R	1/5	6. 0	8. 0	10	5/5	
12	61	−3–58	M66-3R	5/5	11. 4	13. 8	>34	3/5	17. 5	M66-3R	0/3				3/3	
13	96	−3–34	M66-3R	4/5	9. 2	12. 5	>11, >27	0/5	17						0/0	
14	150	−3–34	M66-3R	0/5				0/5	8						0/0	

*C57BL/6 male mice.
†One spleen plus 10 ml N saline for both leukemias.
‡Trocar implant, approximately 25 mg, for M46R and M66-3R.

TABLE 5.—Effect of AM and DCM on the ability of normal spleen to immunize against the growth of C82R-resistant leukemia*

Group	Treatment: Dose (mg/kg)	Treatment: Schedule daily	First period (days −4–6): Inoculum† (day 0)	First period (days −4–6): Survivors Total	Second period (days 7–96): Inoculum‡ (day 7)	Tumor occurrence: Takes Total	Tumor occurrence: Maximum tumor diameter Average (mm)	Tumor occurrence: Maximum tumor diameter (day)	Tumor occurrence: Tumor duration: 2 longest (days)	Survivors: Survivors Total	Survivors: Median day of death
				I. Tumor growth and regression in incompatible host							
1				5/5	C82R	5/5	12.0	7.0	13, 15	5/5	
				III. Lethality with drug treatment of resistant tumor in incompatible host							
2	0.75 AM	−4–42		10/10	C82R	10/10	11.7	7.2	>10	0/10	9
3	56 DCM	−4–42		10/10	C82R	9/10	11.6	7.0	>10, >11	0/10	10
				VII-A. Spleen-induced immunity to tumor inoculation; drug interference with immunity present							
4			Spleen	5/5	C82R	2/5§	6.0	4.5	6, 7	5/5	
5	0.75 AM	−4–42	Spleen	10/10	C82R	10/10	13.0	13.7	>29, 34	3/10	19
6	56 DCM	−4–42	Spleen	10/10	C82R	10/10	12.1	10.4	27, >29	2/10	11.5
				Compatible host controls, CDBA hybrid male mice							
7				5/5	C82R	5/5	12.8	7.2	>10	0/5	9
8	0.75 AM	−4–42		5/5	C82R	5/5	11.2	7.4	>9, >10	0/5	9
9	56 DCM	−4–42		5/5	C82R	4/5‖	12.5	7.0	>9, >10	0/5	9

*Groups 1 to 6, CAF_1 male mice; groups 7 to 9, CDBA hybrid male mice.
†Normal DBA/2J spleen; 5 spleens plus 5 ml N saline.
‡One spleen plus 10 ml N saline.
§Existence of tumors here doubtful; 6 mm palpations were transient and occurred earlier than usual after tumor implant.
‖One mouse dead without tumor 2 days after implant.

TABLE 6.—Effect of schedule of administration of AM on ability of L1210 leukemia to immunize against reinoculation of the disease*

Treatment			First period (days 0–36)						Second period (days 37–63)					
				Tumor occurrence				Survivors		Tumor occurrence				Survivors
Group	AM Dose (mg/kg)	AM Schedule daily	Inoculum† (day 0)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter (day)	Tumor duration: 2 longest (days)	Survivors Total	Inoculum‡ (day 37)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter (day)	Tumor duration: 2 longest (days)	Survivors Total
			I. Tumor growth and regression in the incompatible host											
1								10/10	L1210Z	6/10	9.7	8.7	16	10/10
			I. Tumor growth and regression					and	*IV. Immunity of the incompatible host to reinoculation*					
2			L1210Z	6/10	7.5	8.7	14, 16	10/10§	L1210Z	0/5				5/5
			II. Inhibition by drug of the sensitive tumor					and	*VI. Development of immunity despite drug treatment*					
3	0.37	7–21	L1210Z	4/10	7.2	8.5	11	10/10§	L1210Z	0/5				5/5
4	0.75	7–21	L1210Z	5/10	7.2	7.4	11, 16	10/10§	L1210Z	1/5	11.0	7.0	12	5/5
5	1.50	7–21	L1210Z	0/10				10/10§	L1210Z	0/5				5/5
			II. Inhibition by drug of the sensitive tumor					and	*VI-A. Drug interference with development of immunity*					
6	0.37	1–21	L1210Z	0/10				10/10§	L1210Z	1/5	10.0	7.0	14	5/5
7	0.75	1–21	L1210Z	0/10				10/10§	L1210Z	4/5	10.8	8.0	16	5/5
8	1.50	1–21	L1210Z	0/10				10/10§	L1210Z	2/5	10.0	7.0	12	5/5

*C57BL/6 male mice.

†One spleen plus 30 ml N saline.

‡One spleen plus 20 ml N saline. Inoculum concentration was sufficient to give 100 percent takes in control CDBA hybrid male mice. No titration conducted.

§Five mice from each group killed on day 23 for spleen transplant into CDBA mice. No progressive tumor growth was observed in any CDBA recipients. Recipient mice uniformly succumbed to a subsequent L1210Z tumor inoculation made on day 37.

TABLE 7.—Comparison of effect of AM and DCM on the amethopterin-resistant subline M46R, with and without preinoculation of normal or leukemic spleen suspension*

Treatment			First period (days 0–25)							Second period (days 26–46)						
				Tumor occurrence				Survivors			Tumor occurrence				Survivors	
Group	Dose (mg/kg)	Schedule daily	Inoculum† (day 0)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death	Inoculum‡ (day 26)	Takes Total	Maximum tumor diameter Average (mm)	Maximum tumor diameter Average (day)	Tumor duration: 2 longest (days)	Survivors Total	Median day of death
			I-A. Tumor growth, without regression, in incompatible host													
1								10/10		M46R	10/10	8.7	9.7	>12	0/10	10
			III. Lethality with drug treatment of resistant tumor in incompatible host													
2	0.37 AM	20–40						10/10		M46R	10/10	9.7	10.0	>11, >12	0/10	11
3	0.75 AM	20–40						10/10		M46R	10/10	7.1	9.8	>15	0/10	13
4	1.50 AM	20–40						10/10		M46R	9/10	7.3	10.2	>13, >16	0/10§	12
			III-A. Inhibited tumor growth and decreased lethality with drug treatment of resistant tumor													
5	37 DCM	20–40						10/10		M46R	8/10	7.5	17.5	>20	7/10	17
6	75 DCM	20–40						10/10		M46R	3/10‖	7.0	20.0	>20	7/10	15
7	150 DCM	20–40						10/10		M46R	0/10				0/10§	5
			I. Tumor growth and regression					and		*IV. Immunity of incompatible host to reinoculation*						
8			L1210Z	10/10	9.1	8.3	13, 18	7/10	11	M46R	0/7				7/7	
			I. Tumor growth and regression					and		*V. Retained immunity with drug treatment*						
9	0.37 AM	20–40	L1210Z	10/10	9.9	8.6	15, 18	9/10	11	M46R	0/9				9/9	
10	0.75 AM	20–40	L1210Z	10/10	10.0	8.6	15	10/10		M46R	0/10				10/10	
11	1.50 AM	20–40	L1210Z	10/10	10.0	8.6	14, 18	8/10	9.5	M46R	0/8				0/8§	14.5
12	37 DCM	20–40	L1210Z	10/10	9.8	8.3	13, 15	8/10	11.5	M46R	0/8				8/8	
13	75 DCM	20–40	L1210Z	10/10	10.0	8.0	15, 18	8/10	12.5	M46R	0/8				7/8	5
14	150 DCM	20–40	L1210Z	10/10	9.8	8.3	15, 18	9/10	11	M46R	0/9				0/9§	5

VII. Spleen-induced immunity to tumor inoculation; drug interference with immunity either absent or partial

15				10/10							10/10		
16	0. 37 AM	20–40	Spleen	10/10		M46R	0/10				10/10		
17	0. 75 AM	20–40	Spleen	10/10		M46R	4/10	9. 2	12. 5	>16, 20	6/10	14. 5	
18	1. 50 AM	20–40	Spleen	10/10		M46R	4/10	8. 2	10. 2	>17, 20	1/10§	10	
19	37 DCM	20–40	Spleen	10/10		M46R	0/10				10/10		
20	75 DCM	20–40	Spleen	10/10		M46R	0/10				7/10	15	
21	150 DCM	20–40	Spleen	8/10	26	M46R	0/8				0/8§	5	

*CAF_1 male mice.

†Normal DBA/2J spleen, 7 spleens in 20 ml N saline. L1210Z, 1 spleen plus 10 ml N saline. Leukemic inoculum concentration was 100 times greater than that required to give 100 percent takes in control CDBA hybrid male mice.

‡One spleen plus 30 ml N saline. Leukemic inoculum concentration was 1,000 times greater than that required to give 100 percent takes in control CDBA hybrid male mice.

§Apparent drug toxicity in these groups.

‖Existence of tumors here doubtful; the 7 mm palpations were not observed until long after usual time of occurrence.

Experiment 2. 3′,5′-Dichloroamethopterin suppressed homograft response but failed to alter established immunity

Similar results were obtained in a comparable experiment, also in BALB/c mice, with use of the sensitive L1210 leukemia, the DCM-resistant variant of L1210 leukemia (C82R), and treatment with DCM (table 2).

Section III.—Suppression of the homograft response was evidenced by progressive tumor growth and death of mice inoculated with C82R and treated daily from the day of inoculation with 60 or 75 mg/kg of DCM.

Section I and IV; I and V.—As with the M46R-resistant variant (table 1), after immunization by prior inoculation and regression of the sensitive L1210 or C82R resistant-variant, the C82R reinoculum failed to grow, even when treatment with 60 or 75 mg/kg DCM was administered daily. Treatment with DCM appeared to be somewhat toxic in this experiment as was indicated by the deaths in the second period of animals showing no evidence of tumor growth.

Experiment 3. (*a*) 3′,5′-Dichloroamethopterin suppression of homograft response influenced by time of initiation of therapy (table 3)

Section III.—As in the previous experiment (table 2), treatment with 60 mg/kg of DCM, initiated on the day of inoculation of the C82R-resistant leukemia in BALB/cAn mice, resulted in suppression of the homograft response which was evidenced by progressive tumor growth and death of the animals (group 6).

The ability of DCM to abrogate the homograft response was diminished when the C82R-resistant tumor was not treated until the local tumor at the site of inoculation had become palpable. With this delayed treatment, only 4 of 10 animals succumbed. In the survivors, the tumor regressed during therapy.

Experiment 3. (*b*) 3′,5′-Dichloroamethopterin did not interfere appreciably with the development of immunity (table 3)

We considered it desirable to determine what influence folic acid antagonists might have on the establishment of immunity. For this purpose, treatment initiated in the first period was discontinued prior to reinoculation in the second period.

Section II and VI.—Suppression of the homograft response to a resistant variant appears to be more readily accomplished than interference with the mechanism of development of immunity. The same dose, 60 mg/kg, and schedule of DCM that resulted in progressive tumor growth and death in mice with the resistant tumor (section III, group 6) failed to interfere substantially with the ability of an initial inoculum of L1210 to render the mice immune to a second inoculation with the C82R variant. Immunity resulted even though treatment with DCM appeared to inhibit completely the growth of the L1210 leukemia.

With delayed therapy, the L1210 tumor also regressed and the animals were immune on reinoculation with the C82R variant.

The 6 animals with C82R that survived after delayed treatment with AM (group 7) were also immune to reinoculation of the C82R tumor.

Section VII.—Treatment with 60 mg/kg of DCM, initiated on the day of inoculation of normal spleen, also failed to interfere with the ability of spleen to immunize the mice against subsequent inoculation of the C82R-resistant tumor. However, on delayed administration of DCM to mice inoculated with spleen, transient tumor growth was observed in 7 of 10 mice after reinoculation with C82R. The reinoculum-control group (section I) showed 8 of 12 deaths.

Experiment 4. Low doses of amethopterin and 3,′5′-dichloroamethopterin did not interfere appreciably with the homograft response or development of immunity

In experiment 3, a dose level of DCM that resulted in progressive growth of the C82R-resistant leukemia failed to interfere with the ability of an inoculum of L1210 or spleen to immunize the mice to reinoculation of the C82R leukemia. Also, survivors of L1210 and C82R, after delayed administration of DCM, were immune to reinoculation of the C82R leukemia. In similar manner, dose levels of antifolic too low to suppress the homograft response with resistant variants did not interfere extensively with the development of immunity (table 4). In this experiment, dose-response curves were obtained with AM against the M46R-resistant variant and with DCM against the M66-3R-resistant variant, in C57BL/6 mice.

Section I.—Growth and regression of the tumors were observed with both the M46R and M66-3R controls.

Section III and V, VI.—The abrogation of the homograft response appeared to be related to drug dosage. At the lower dose levels, for both drugs, the homograft response was not appreciably suppressed, and all animals survived. At somewhat higher dose levels the local tumors at the site of inoculation were larger than in the untreated controls, and subsequent regression of the local tumor was either delayed or prevented. Many animals succumbed to tumor growth, deaths occurring a week or more after the homograft response appeared in the controls. At the highest dose of DCM administered, tumor growth was completely inhibited, but most of the animals died with marked loss of weight.

In those mice in which treatment did not overcome the homograft response, reinoculation with the resistant tumors resulted in a reduced incidence of tumor growth. The appearance of immunity was manifested by a decrease in tumor size during treatment and by failure of the resistant tumor reinoculum to grow, in many instances, despite the continuation of therapy.

Experiment 5. Extensive treatment with folic acid antagonists may interfere with ability of spleen to immunize (table 5)

Section VII-A.—As previously shown, for BALB/cAn mice (table 3), preinoculation of spleen interfered with the ability of C82 to grow in CAF_1 mice. However, continuous treatment with AM or DCM, initiated 4

days prior to inoculation of spleen, reduced the inhibitory effect of spleen, and we noted the progressive growth and death of mice subsequently inoculated with the C82R tumor. The diminution, by treatment, of the ability of spleen to immunize was not quite complete. This was more evident with AM than with DCM. Mice treated with AM and then inoculated with spleen and the C82R leukemia showed progressive tumor growth and death (group 5). However, they lived longer and there were more survivors than in the group receiving AM and an inoculum of C82R (section III, group 2). There was a 19-day median survival time and 3 of 10 survivors, as contrasted with a 9-day median survival time and 0 of 10 survivors. With DCM, inoculation of spleen increased the median survival time by 1.5 days and 2 of 10 animals survived (group 6).

Experiment 6. Treatment with folic acid antagonists may also interfere to some extent with the ability of L1210 leukemic spleen to immunize; effect of treatment schedule

This experiment was conducted in C57BL mice.

Section I, and I and IV.—In the untreated controls, transient tumor growth was observed in 60 percent of the animals. In the reinoculated controls 0 of 5 takes occurred.

Section II and VI-A.—With treatment at 3 different levels of AM from day 1 to 21, none of the mice grew palpable tumors. In contrast to the reinoculated controls, 7 of 15 takes occurred on reinoculation.

Section II and VI.—Mice not treated until 7 days after inoculation of leukemic spleen suspension were immune to reinoculation.

Experiment 7. 3′,5′-Dichloroamethopterin inhibited progressive growth of the M46R variant; antifolic therapy may interfere to some extent with already established immunity

In this experiment, spleen was inoculated on day 0, treatment given from day 20 to 40, and M46R inoculated on day 26 (table 7).

Section I-A.—The M46R inoculum in this experiment was extremely effective and resulted in progressive tumor growth and death in all the CAF_1 mice.

Section III.—By the same token, progressive tumor growth and death occurred at the 3 dose levels of AM.

Section III-A.—Treatment of the M46R variant with DCM had an inhibitory effect on the growth of the resistant tumor at the 2 highest doses, 150 and 75 mg/kg daily. At the lowest dose (37 mg/kg daily), transient tumor growth was observed in most animals. Except at the highest dose of 150 mg/kg, which was toxic, an appreciable number survived. This inhibition appears to reflect that the M46R tumor is partially susceptible to the tumor-inhibitory action of DCM.

Section VII.—Preinoculation of spleen suspension prevented growth of the M46R variant. However, at the 2 highest doses of AM (0.75 and 1.5 mg/kg daily), treatment appeared to overcome partially the immunity conferred by spleen, and a number of takes resulted.

After preinoculation of normal spleen and treatment with DCM, we observed inhibition of growth of the M46R reinoculum.

Section I and V.—After preinoculation of leukemic spleen, treatment with AM or DCM failed to overcome the established immunity.

DISCUSSION

These studies in homologous mice show that the observation of suppression of the homograft response to resistant variants of L1210 leukemia, when the mice are treated with folic acid antagonists, is not at variance with the finding that successful therapy of L1210 in the compatible host results in the elicitation of an immune response.

It is interesting to compare the previously reported results in compatible ("semi"-isologous CDBA) mice (*1–3*) with our results in incompatible (homologous) strains.

For compatible, spontaneous regression of the L1210 leukemia or its variants has not been observed. Mice surviving systemic L1210 leukemia after treatment with DCM were immune to reinoculation of the sensitive or resistant leukemia (*1–3*). Preinoculation of the mice with the sensitive leukemia augmented therapy with DCM of an antifolic-resistant variant (*5*). Apparently, treatment of systemic L1210 leukemia results in elicitation of host immunity that contributes to the therapy of the resistant variant. Augmentation of therapy of the resistant variant did not occur if treatment was discontinued too soon.

For incompatible strains, spontaneous regression of the leukemia and its variants occurred. Treatment was not necessary to induce regression. After preinoculation and spontaneous regression of the sensitive L1210 leukemia, or the resistant variants, the mice were immune to reinoculation of the sensitive or resistant sublines.

On administration of folic acid antagonists to both compatible and incompatible strains, the resistant variants grew progressively and death of the animals resulted.

As previously indicated, successful treatment of systemic sensitive leukemia with DCM, in the compatible host, resulted in the development of immunity. In homologous strains, although antifolic therapy abrogated the homograft response, at therapeutic doses it did not interfere appreciably with the development of immunity. Both in the "semi"-isologous (*4*) and homologous strains, once immunity is established, it is not too readily suppressed by antifolic therapy.

The CDBA mice could not be immunized with DBA/2J or CDBA spleen or with leukemic inoculum levels that failed to result in takes (*2*). Even though L1210 leukemia has been carried for over 10 years, lack of histocompatibility of tumor and host is apparently no more than minimal (*14*). Inability of the antifolics to act against the tumor cells, rather than any suppression of immune response, appears to account for the failure of therapy against the antifolic-resistant tumors in the compatible host.

In homologous mice, the resistant variant cells evidently remain resistant to antifolic therapy. Since the antifolic therapy interferes with homograft response, the animals succumb to tumor growth.

Thus, a major difference in response in isologous as compared with homologous mice appears to pertain to the homograft response in the homologous mice, which results from histocompatibility differences of tumor and host. The suppression of the homograft response to resistant variants of L1210 leukemia by antifolics in homologous mice corroborates previous reports (*7*, *8*). The suppression of the homograft response was more readily obtained when treatment was initiated prior to or at the time of inoculation of the resistant tumor. A dose-response relationship was observed with respect to suppression of the homograft response. At low doses the resistant tumors regressed spontaneously.

The extent of suppression of homograft response may also be subject to the degree of resistance of the tumor. The M46R variant is quite resistant to treatment with AM and there was marked abrogation of the homograft response with this drug. The tumor variant is, on the other hand, partially sensitive to DCM and with this drug there was marked inhibition of tumor growth. With DCM, therapy alone, or immunity, or both acted to retard the growth of the tumor.

Several possible mechanisms could account for the progressive tumor growth and death with antifolic treatment of resistant tumor in the incompatible host:

(*a*) The drug exerts host toxicity and the animals succumb to drug toxicity alone or to a combination of drug toxicity and tumor growth. This would not account for the observation of delayed tumor resorption and delayed death with therapy.

(*b*) The resistant tumors are also dependent upon the folic acid antagonist for optimal growth. In this case, the tumor would grow slowly without treatment and more rapidly with treatment. This appears to be unlikely since there was no definite evidence of actual stimulation of tumor growth by the antifolics. Also, toxic doses or a more effective congener can interfere with growth of a resistant tumor.

(*c*) Suppression of the host-immune mechanism can permit progressive growth of the resistant leukemia. Also, with treatment, partial sensitivity of a resistant tumor could result in slower growth and altered antigenic effect. Studies are in progress on the relationship of the antigenic properties of tumor inoculums to the development of host immunity with and without therapy.

If the observation that antifolic therapy may overcome the homograft response to resistant variants, with progressive tumor growth and death of the animals resulting, were a reflection of a generalized suppression by antifolics of immune responses, the use of the drugs could, in certain situations, be contraindicated. However, the drugs failed to interfere substantially with the development of host immunity in the compatible or incompatible strains of mice, or to suppress, appreciably, already established immunity. As we have indicated, failure to interfere with

the growth of the resistant tumor in the compatible strain appeared to be attributable to tumor-cell resistance to the drugs, rather than to suppression of any immune mechanism in the host.

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Intrasplenic Transplantation of Neonatal Thymus [1, 2]

ROGER W. O'GARA *and* JEWEL ARDS, *Laboratory of Pathology, National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

Transplants of thymus from neonatal C57BL mice into the spleens of adult C57BL mice grew equally well in thymectomized and intact animals. Studies of early intrasplenic thymic grafts showed that, although there was marked depletion of the thymocytes during the first 4 days after transplantation, groups of transplanted thymocytes persisted and completely repopulated the graft by the 7th day after transplantation. In contrast, subcutaneous transplants of neonatal thymic tissue grew more slowly, showed greater depletion of thymocytes, and were not entirely repopulated until the 11th day. Intrasplenic thymic grafts persisted and were identified in almost half the mice for as long as 24 months after transplantation. The transplanted thymocytes could be distinguished morphologically from the lymphocytes of the spleen by their greater hyperchromasia and sharper staining with hematoxylin. One cystic squamous-cell carcinoma, several epithelial cysts lined by columnar cells, and 2 hemangiomas arose in the spleen at the site of the transplanted thymic tissue.—J. Nat. Cancer Inst. 27: 277-297, 1961.

THE THYMUS has been the subject of numerous investigations in the past, but many questions concerning its morphology and function remain unanswered. Particular attention has been given to a possible hormonal function of the thymus and to the origin of thymocytes and thymic epithelium (or thymic reticulum). Several excellent reviews have concluded that extirpation of the thymus produces no detectable alteration in animals (*1*, *2*), and that administration of either whole thymus or extracts of thymus has had no appreciable effect on the physiology or development of experimental animals (*2*, *3*). Recently, however, there have been additional reports that a lymphocyte-stimulating factor (*4*) and a *d*-tubocurarine-like substance (*5*) have been found in the thymus.

Methods of investigation used in the past have included ablation of the thymus and transplantation to various sites (*6–10*). Because of reports on the development of neoplasms after intrasplenic transplantation of ovaries and testes in gonadectomized animals (*11–13*) and of thyroid in thyroidectomized animals (*14*), experiments were undertaken to transplant

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the thymus from newborn C57BL mice to the spleens of older mice of the same strain with and without thymectomy. If neoplasms developed, this might suggest that the thymus, like the ovary, is an organ of internal secretion regulated by a feed-back mechanism which is dependent upon reciprocal inhibition by its own secretions. An opportunity would also be afforded to observe the histogenesis of such neoplasms and to compare them with thymic tumors in man (*15–18*). In any event, the behavior and morphologic changes of the thymus graft within the confined space of the spleen could be more readily observed than in the subcutaneous tissue. Although no hormonal effect was demonstrated, one transplantable tumor and several cysts arose at the site of the graft, and information on the growth and involution of thymic grafts in the spleen was obtained.

MATERIALS AND METHODS

Male and female C57BL/6JN mice were thymectomized at 5 to 6 weeks of age by aspiration of the thymus through a glass medicine dropper attached to a house vacuum line. The tip of the glass tube was inserted into the anterior mediastinum through a midline vertical incision in the skin of the neck and in the underlying sternohyoid muscles.

Two to 7 days after thymectomy, some of the animals received an intrasplenic graft of one lobe of thymus (1–2 mm in greatest diameter) from 1- to 3-day-old C57BL mice. The thymic tissue used for transplantation was dissected from the mediastinum of donor mice under aseptic conditions and placed in a sterile solution of physiological saline. An effort was made to separate the thymus from all adjacent tissue, and usually transplantation was made within one-half hour after removal of the thymus from the donor mouse. The spleen of the recipient mouse was delivered through a midline incision in the abdomen, and the thymic tissue inserted under the capsule of the spleen with an 18-gauge steel trocar. Sham operations were performed in the same manner, except that an empty trocar was used.

The animals were killed after a period of 1 day to 2 years and all the organs except the brain were examined post mortem. Tissues were fixed in Zenker-acetic acid. After fixation, the spleens were bisected longitudinally and examined for the presence of the transplanted tissue. All the tissues were then embedded in paraffin and stained routinely with hematoxylin and eosin. Additional sections of the spleen were stained for iron by Gomori's Prussian-blue method and impregnated for reticulum by the Wilder method.

RESULTS

Histologic Study of Early Thymic Transplants

The morphologic changes in the thymic graft after transplantation into the spleen or subcutaneous tissue were studied. A total of 121 male C57BL mice (42 thymectomized and 79 intact) was killed at intervals

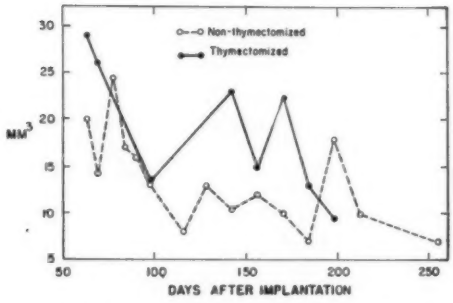
from 1 to 255 days after transplantation of thymus into the spleen, and observations were made of the size and histologic appearance of the graft. A similar study was made of subcutaneous thymic grafts in 41 intact male mice killed 1 to 29 days after transplantation. A quantitative evaluation of some of the observations during the first 14 days is given in table 1.

TABLE 1.—Regeneration of intrasplenic and subcutaneous transplants of neonatal thymus*

Day	Nonthymectomized						Thymectomized	
	Subcutaneous graft			Intrasplenic graft			Intrasplenic graft	
	Number of grafts	Number of thymocytes	Relative No. of large thymocytes	Number of grafts	Number of thymocytes	Relative No. of large thymocytes	Number of grafts	Number of thymocytes
1	2	1+	±	2	2+	±		
2	2	2+	±	2	1+	4+	2	2+
3	4	1+	+	3	2+	3+		
4	6	2+	+	6	3+	3+	2	1+
5	3	2+	3+	2	4+	2+		
6	3	2+	3+	1	2+	+	2	3+
7	2	3+	2+	2	4+	±		
8	2	3+	+	1	4+	±	2	4+
9	2	3+	±	2	4+	±		
10	2	3+	±	2	4+	±	2	4+
11	2	4+	±					
12	2	4+	±	2	4+	±	2	4+
13	2	4+	±					
14	2	4+	±	2	4+	±	2	4+

*Subcutaneous grafts consisted of a whole thymus from a neonatal mouse. Intrasplenic grafts were made of a single lobe of thymus.

Intrasplenic transplants of thymic tissue were usually easily visible grossly after the 6th day in both thymectomized and intact mice, and showed no appreciable differences during the first 2 months (figs. 1, 2, and 3). For the first 3 days the graft consisted chiefly of elongated or spindled reticular cells (figs. 4 and 5). Thymocytes were scarce, but groups of these cells were found consistently at the margin of the grafts (fig. 4A). During this period, many thymocytes were larger and less hyperchromatic than the mature thymocyte (fig. 5A). Numerous mitotic figures were associated with mixed groups of large and small thymocytes, and when regeneration occurred it was from these groups of cells at the margins of the graft. The transplanted thymic tissue remained discrete, and there was no evidence of infiltration by lymphocytes from the host. By the 6th day the large thymocytes had largely disappeared, and the grafts were completely repopulated with mature thymocytes (figs. 6 and 6A). After the 6th day the histologic structure of the graft resembled that of the normal thymus (fig. 7). Intrasplenic thymic transplants in animals autopsied 2 to 6 months after transplantation were usually slightly larger in thymectomized mice than in intact ones (text-fig. 1), but histologically they were identical.



TEXT-FIGURE 1.—Relative size of intrasplenic thymic transplants in thymectomized and nonthymectomized C57BL mice. Obtained by multiplying together the linear measurements in three dimensions. Each point represents the average for 2 mice.

Early subcutaneous transplants with one lobe of thymus were difficult to locate; therefore a whole newborn thymus was transplanted instead of only one lobe. Inflammatory infiltrates of lymphocytes and granulocytes were frequently found about the graft or at the site of transplantation. Reticular cells were the most frequent in the graft during the first few days, but groups of thymocytes were found consistently in all subcutaneous grafts (table 1). Large thymocytes began to appear in the graft about the 4th day and were associated with an increase in mitotic figures. The large thymocytes persisted until the 8th day, after which they were replaced by the smaller hyperchromatic thymocytes. No evidence was found of regeneration of thymocytes from the reticular cells of the graft. The subcutaneous grafts therefore differed from the intrasplenic grafts in a greater depletion of thymocytes, a slower repopulation of the graft, and the presence of inflammatory cells.

Histologic Appearance of Older Thymic Transplants

A second group of thymectomized C57BL mice was allowed to live to the age of 2 years unless they became moribund or died earlier (table 2). Eighty-four of 92 mice were in sufficiently good condition for satisfactory autopsy. Fifty-four had received an intrasplenic graft of thymic tissue from neonatal mice, 24 a sham transplant into the spleen, and 6 a transplant of thymic tissue into the pancreas at the hilus of the spleen.

Survival of transplants.—After a year or more, thymic tissue transplanted into the spleen was identified in 14 of 31 male and in 8 of 23 female thymectomized animals—45 and 35 percent, respectively (table 2). Identification was doubtful in 4 mice and these were considered negative. The grafts were more easily identified in animals autopsied immediately after death, and of 23 male and 19 female animals killed, grafts were recovered in 57 percent of the males and 37 percent of the females. This proportion of recoveries for males is comparable to that obtained with transplants

TABLE 2.—Neoplasms in thymectomized C57BL mice after transplantation of thymic tissue from neonatal mice

Sex and number of mice	Implants identified at autopsy	Average age at death (months)	Location of tumor*	
			At site of graft	Lymphoid tissue
Thymectomized, intrasplenic graft†				
M 31	14	22.7	Hemangioma (1) Squamous-cell carcinoma (1) Respiratory epithelium and cysts (4)	Reticular-cell sarcoma (1) Leukemia (1)
F 23	8	22.3	Hemangioma (1) Respiratory epithelium (1)	Reticular-cell sarcoma (3)
Thymectomized, intrapancreatic graft†				
M 6	4	24.0	Respiratory cysts (3)	0
Thymectomized controls (intrasplenic sham graft)†				
M 9	—	21.9	0	0
F 15	—	23.9	0	Reticular-cell sarcoma (3) Leukemia (1)
Intact controls (untreated)				
M 5	—	18.8	—	0
F 13	—	21.5	—	0

*Number of tumors in parentheses.
†Mice were 2 months old at the time of transplantation.

into the pancreas in a small group of males, all of which were examined immediately after killing, and is probably the highest proportion obtainable unless serial sectioning is used.

Gross.—The thymic transplants were visible on gross examination as white nodules beneath the capsule of the spleen in 11 mice (fig. 8). In 11 other mice, the transplants were identified only on microscopic examination of the stained tissue. The transplants usually measured from 1 to 3 mm in greatest diameter, though occasionally larger ones were seen with diameters of 4 to 7 mm. The more deeply placed nodules were more difficult to find than those located close to the capsule.

Microscopic.—Most grafts formed small, circumscribed nodules composed chiefly of thymocytes with a few islands of reticular or epithelial cells (figs. 9, 10, and 11). The thymocytes could be distinguished morphologically from the lymphocytes of the spleen by the greater hyperchromasia of their nuclei. In general the nuclear chromatin was more sharply outlined in the thymocytes, and it took a bluish-black stain with hematoxylin that contrasted with the dull black of the lymphocytic nuclei (figs. 11A and 11B). Often a small knot of chromatin was seen near the center of the thymocyte nucleus and this was less frequent in the nucleus of the lymphocyte. Stroma was scant in the transplants and the thymocytes were more closely packed than were the lymphocytes in the

follicles of the spleen. Occasionally a transplant was partly surrounded by a thin layer of fibrous tissue. The transplants, in most cases, had a considerable number of reticular fibers, which helped to identify the graft. Reticular fibers, on the other hand, were scant in the spleen except for a few near blood vessels and about the periphery of the lymphoid follicles.

In younger animals, deposits of brownish pigment which stained for iron were found in macrophages adjacent to many transplants. In older animals, however, much of this pigment failed to stain for iron, and accumulations of iron-positive pigment elsewhere in the spleen made the presence of iron pigment less specific. In older animals, also, deposits of amyloid often accumulated about the margins of the lymphoid follicles in the spleen while the thymic graft remained free of amyloid (fig. 10). In many mice a small amount of adipose tissue was found with the thymic graft in the spleen. The fat cells presumably arose from thymic lipoblasts after partial atrophy of the thymus, though the possibility that they came from adipose tissue included with the graft cannot be ruled out.

Tumors and cysts.—One malignant neoplasm was found in the spleen. This tumor was a large, cystic carcinoma composed of basal and squamous epithelial cells (fig. 12). To date, this tumor has been successfully carried for 10 transplant generations. The first transplant grew slowly and had a latent period of 7 months. Recent transplants, which are predominantly squamous carcinomas, have grown more rapidly, with a latent period of about 3 weeks. Ulceration of the skin over the tumor occurs frequently. In 3 mice, a group of ducts embedded in fibrous tissue (fig. 13) or fragments of glands associated with cartilage were found, and in 2 mice fragments of striated muscle were noted in the graft. Cysts lined by columnar epithelium and with cartilage in the wall (fig. 14) were found in the spleens of 2 animals. Three of 6 implants in the pancreas also showed cysts lined by columnar epithelium, 2 of which had cartilage in the wall.

Additional Tumors

One lymphocytic leukemia and 4 reticular-cell sarcomas occurred in thymectomized mice bearing intrasplenic transplants, and 1 leukemia and 3 reticular-cell sarcomas in mice with sham transplants (table 2). None of these neoplasms involved the transplanted tissue. Hemangiomas were found in 3 thymectomized mice bearing transplants; 2 mice had solitary hemangiomas in the spleen at or near the site of transplantation, and 1 had multiple hemangiomas of the liver, paw, and cervical lymph nodes. No hemangiomas were observed in mice with sham transplants.

Adenomatous polyps of the colon, jejunum, and duodenum were found in 5 thymectomized mice, 21 to 24 months of age (4 males and 1 female); none occurred in intact mice. Duodenal polyps of the Brunner-gland type, which have been described in DBA mice (*19*), occurred in 35 percent of all the mice 19 months of age or older, both experimental and control. These intestinal polyps are omitted from table 2.

DISCUSSION

These experiments demonstrate that thymic tissue from newborn C57BL mice will persist and maintain its structural integrity for at least 2 years when transplanted into the spleen of young adult mice of the same strain. Thymocytes largely disappear from the central portion of the graft during the first 3 days after transplantation, but, at the margin, small groups of thymocytes persist and within a week completely repopulate the graft. Our results with subcutaneous grafts suggest that the thymocytes in the subcutaneous grafts, like those in the intrasplenic graft, regenerate from the surviving thymocytes of the original transplant. This sequence differs from that reported after subcutaneous transplantation of thymic tissue (*9*, *20*). Gottesman and Jaffe (*9*), using intramuscular autologous transplants of adult rat thymus, reported almost complete disappearance of thymocytes during the first 4 days after implantation. After 4 days the thymocytes reappeared, apparently arising from the reticular cells of the thymic transplant. Carnes *et al.* (*20*), using subcutaneous transplants of isologous adult thymus in irradiated and nonirradiated mice, noted disappearance of the thymocytes of the graft on the 7th day after transplantation and evidence of regeneration of thymocytes from the reticular cells of the graft by the 10th day. No description is given of the appearance of the graft immediately before or after the 7th day. Possibly our use of neonatal thymic tissue, which has a greater growth potential, allowed more thymocytes to survive the transplantation procedure. It is also probable that the small size of the transplanted neonatal thymus would tend to minimize the destruction of cells due to disturbed nutrition during the critical period after transplantation. It is certain, however, that the degeneration of thymocytes in subcutaneous grafts is more severe and prolonged than it is in intrasplenic grafts. The difference in reaction may be due to the greater vascularity of the spleen as compared to the subcutaneous tissue, although other factors, such as differences in temperature, more confined space, or presence of hematopoietic tissue in the spleen may also play a part.

The histologic appearance of older intrasplenic grafts resembled that of the normal thymus, and none of the histologic patterns of epithelial thymomas in man or of lymphocytic thymomas in mice or man were produced. The columnar epithelial cysts found in the thymic grafts probably arose from the small cysts lined by ciliated columnar epithelial cells, which are frequently seen in the thymus of the mouse. Alternatively, they may have arisen from fragments of trachea accidentally transplanted with the thymus. The columnar epithelial cysts within the thymus transplant appeared benign, both on gross and on microscopic examination, and no transplantations were attempted. The squamous-cell carcinoma presumably arose from squamous cells derived from the thymic epithelium within the intrasplenic thymic graft. Although Hassall's corpuscles are not well developed in the thymus of the mouse, there are usually many small whorls of noncornified epithelial cells which probably represent

Hassall's corpuscles. Microscopically in this carcinoma, both the squamous epithelial cells lining the cyst and the basal cells appeared malignant. On subcutaneous transplantation this tumor grew as a squamous-cell caricinoma in 1 of 3 mice after a latent period of 7 months. The tumor is now in its tenth transplant generation, and grows in 100 percent of transplanted animals with a latent period of about 3 weeks. In the tumor-bearing hosts no specific changes have been observed which could be attributed to a humoral effect of the tumor.

Differences were noted in the morphology and staining characteristics of nuclei in thymocytes and lymphocytes. The thymocytes in the intrasplenic graft closely resemble the thymocytes in the intact thymus, and it may be that the epithelial cells or stroma of the thymus influences the appearance of the thymocyte. On the other hand, thymocytes appear to be functionally different from lymphocytes of the spleen and lymph nodes, as has been shown by experiments on antigenic stimulation in animals (*21*).

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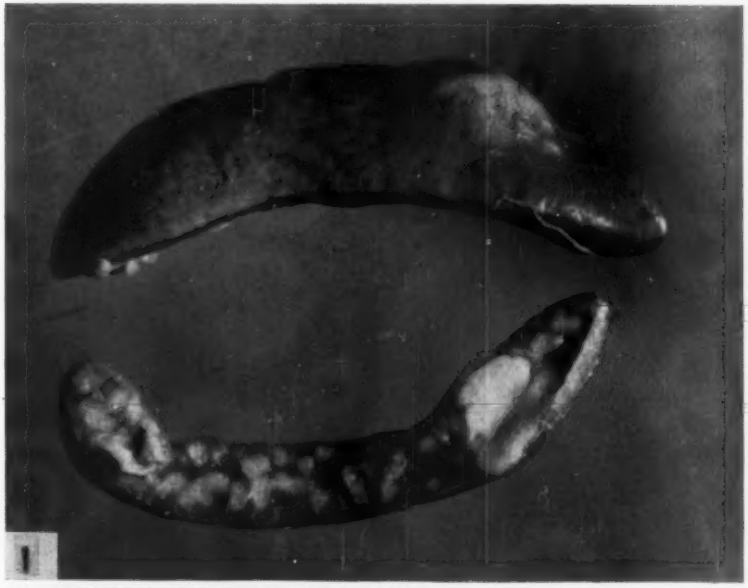
PLATES

PLATE 31

FIGURE 1.—Intrasplenic thymus transplant in thymectomized mice after 12 days. One spleen has been sectioned longitudinally through the graft; other spleen is intact. × 6.5

FIGURE 2.—Intrasplenic thymus transplant in a nonthymectomized mouse after 12 days. × 6.5

FIGURE 3.—Intrasplenic thymic transplants in mice after 98 days (fixed in Zenker's acetic acid). Two spleens on *right* are from thymectomized animals and two on *left* are from nonthymectomized animals. Upper spleen in each set has been sliced longitudinally to show internal appearance. × 2.6



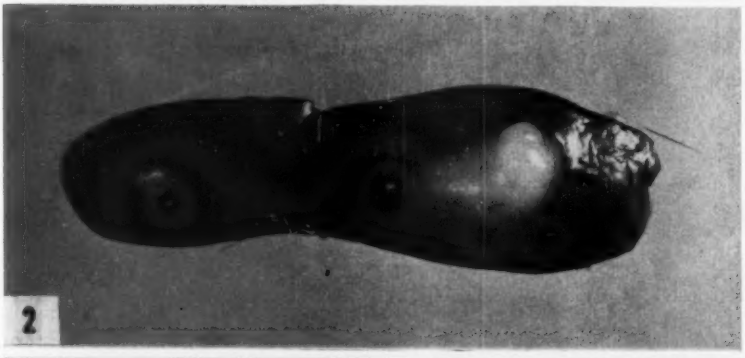


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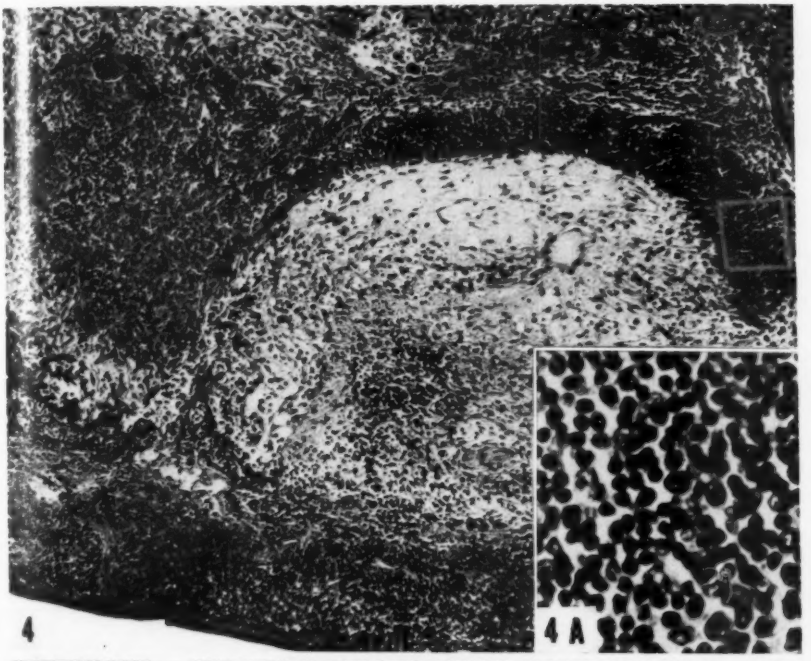
PLATE 32

FIGURE 4.—Intrasplenic thymic transplant in thymectomized mouse after 2 days. Demilune of surviving thymocytes is seen *above* at margin of central necrotic area. Small nodule of thymic epithelial cells is seen in *upper left*, and small amount of carbon-black pigment, injected with graft, is present just below the epithelial cells. Hematoxylin and eosin. × 115

FIGURE 4A.—Higher magnification of area outlined in white. Most thymocytes are small and dark-staining. × 520

FIGURE 5.—Intrasplenic thymic transplant in nonthymectomized mouse after 3 days. Nodule is surrounded by relatively acellular zone. Central necrotic area is partly surrounded by a band of viable thymic reticular or epithelial cells, and there is a dense band of thymocytes at *upper margin*. Small cystic structure lined by cuboidal cells is seen in *upper right*. Similar cysts are often seen in sections of normal thymus. Hematoxylin and eosin. × 100

FIGURE 5A.—Higher magnification of area outlined in white. Many large, pale thymocytes are seen among smaller darker-staining ones. × 520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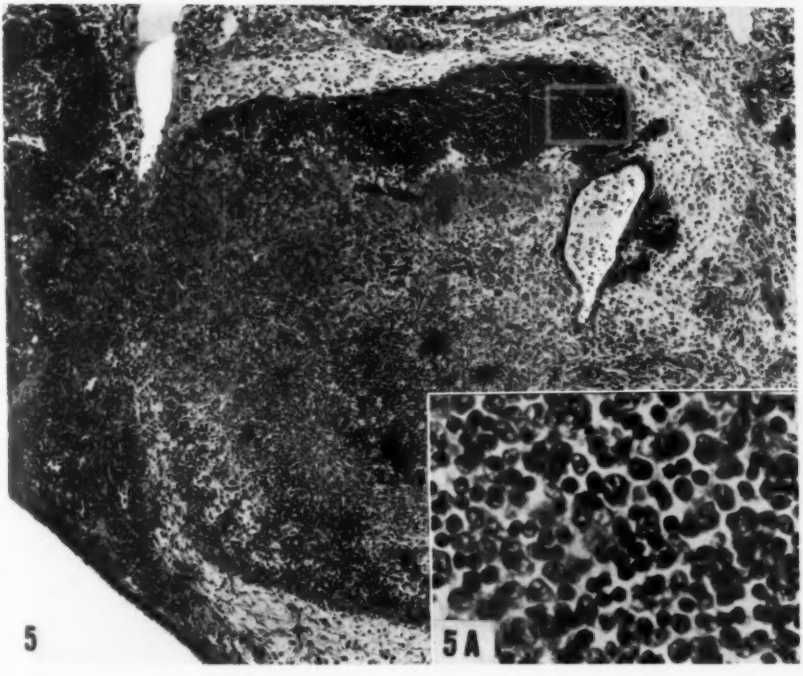
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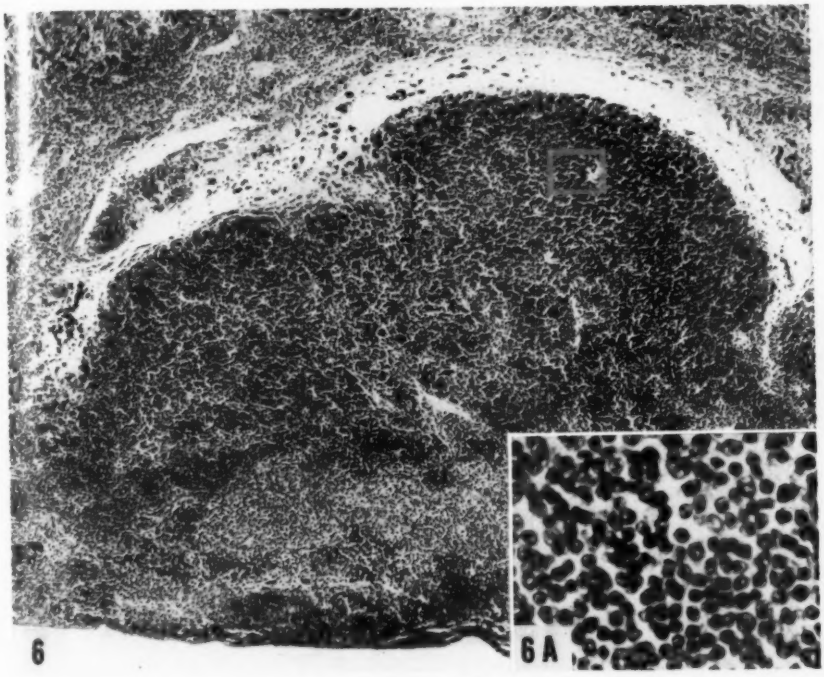
PLATE 33

FIGURE 6.—Intrasplenic thymic transplant in thymectomized mouse after 6 days. Graft is still partly surrounded by acellular zone which contains scattered particles of carbon black injected with graft. Nodule has definite cortex and medulla, though not as distinct as in normal thymus. Hematoxylin and eosin. × 85

FIGURE 6A.—Higher magnification of area outlined in white. Most thymocytes are again small and dark-staining as they were 2 days after transplantation. × 520

FIGURE 7.—Intrasplenic thymic transplant in nonthymectomized mouse after 22 days. Implant remains discrete and separate from splenic tissue. Pale areas within graft are islands of thymic reticular cells. Hematoxylin and eosin. × 50



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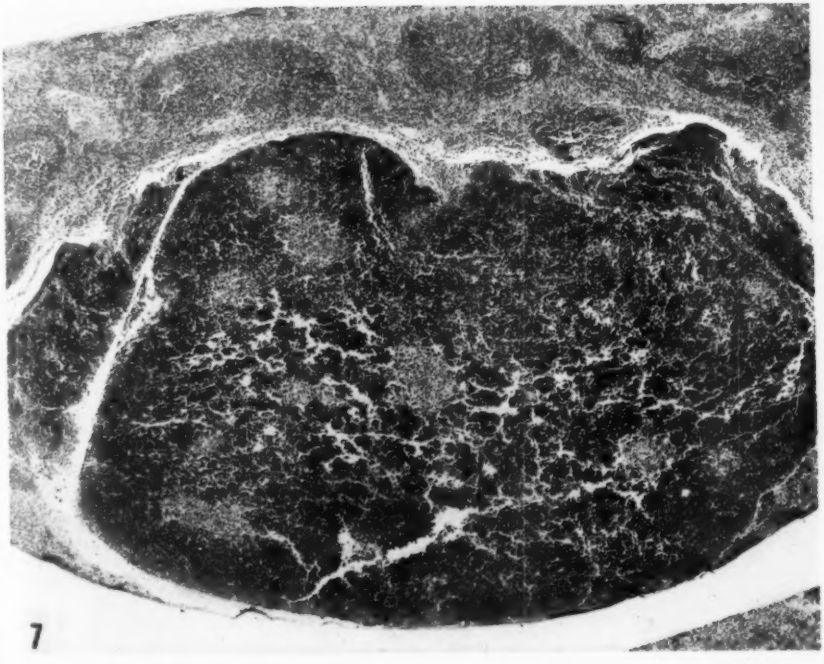

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PLATE 34

FIGURE 8.—Spleen 23 months after intrasplenic transplantation of neonatal thymic tissue. Thymic tissue can be seen as white subcapsular nodule 2 mm in diameter. × 3.3

FIGURE 9.—Intrasplenic thymus transplant after 23 months in spleen. Closely packed thymocytes stain darkly and occupy most of central portion of spleen. Pale areas within graft are islands of thymic reticular cells. Splenic lymphoid follicles (adjacent to capsule) stain less intensely and are more loosely packed. Hematoxylin and eosin. × 50

FIGURE 10.—Intrasplenic thymus transplant after 24 months in spleen. Closely packed, darkly staining thymocytes are seen at *bottom* along with a few fat cells. Splenic lymphoid follicles at *top* are surrounded by ring of amyloid which is usually deposited first in this location. Hematoxylin and eosin. × 62

8

9

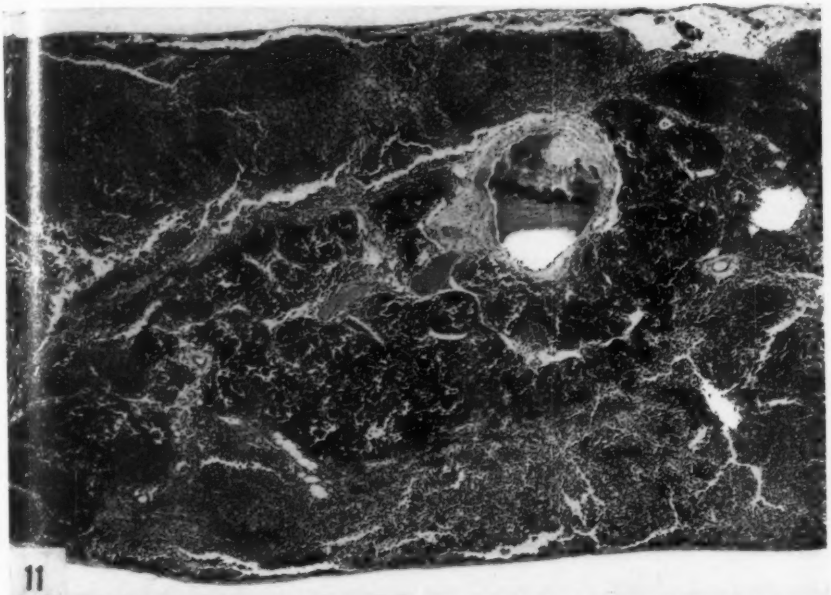
10

PLATE 35

FIGURE 11.—Intrasplenic thymus transplant after 24 months in spleen. *Compare* appearance of graft (in *center*) with that of splenic lymphoid follicles adjacent to capsule. Large blood vessel is seen within graft. Hematoxylin and eosin. × 50

FIGURE 11A.—Higher magnification showing appearance of transplanted thymic cells. Hematoxylin and eosin. × 520

FIGURE 11B.—Higher magnification showing appearance of lymphocytes from adjacent splenic follicle. Hematoxylin and eosin. × 520



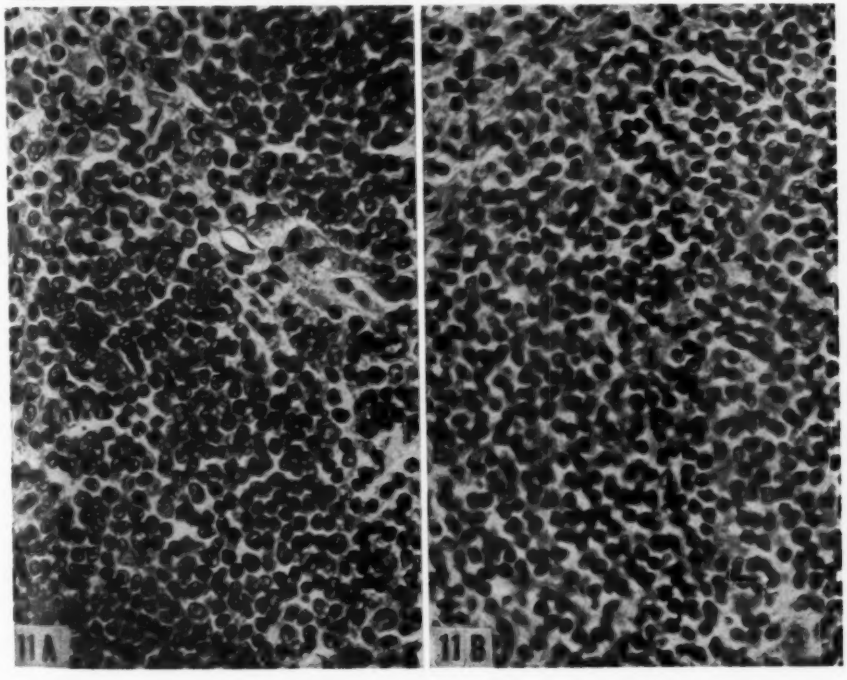


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PLATE 36

FIGURE 12.—Intrasplenic transplant of thymus after 24 months. Spleen contains large cystic tumor lined by stratified squamous epithelium. There is marked proliferation of basal cells and keratinization with formation of numerous epithelial pearls. Portions of this tumor are being carried serially as transplantable squamous-cell carcinoma. Hematoxylin and eosin. × 70

FIGURE 13.—Intrasplenic transplant of thymus after 19 months. Group of ductlike structures lined by low-columnar epithelial cells. Ducts are surrounded by dense fibrous tissue. Most of splenic tissue has been replaced by amyloid deposits. Hematoxylin and eosin. × 75

FIGURE 14.—Intrasplenic transplant of thymus after 23 months. Spleen contains large cyst lined by low-columnar epithelial cells and supported by thin layer of collagen. A few fragments of hyalin cartilage present in wall give it appearance of bronchial cyst. A few clumps of dark-staining cells resembling thymocytes are present in the adjacent tissue. Hematoxylin and eosin. × 60

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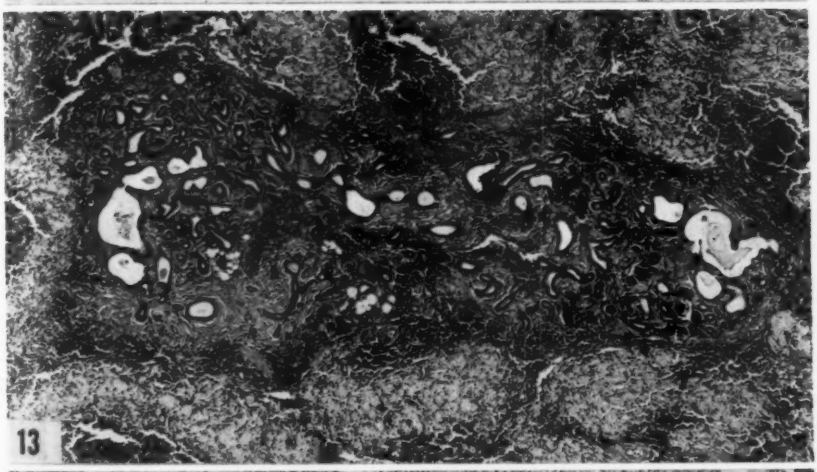
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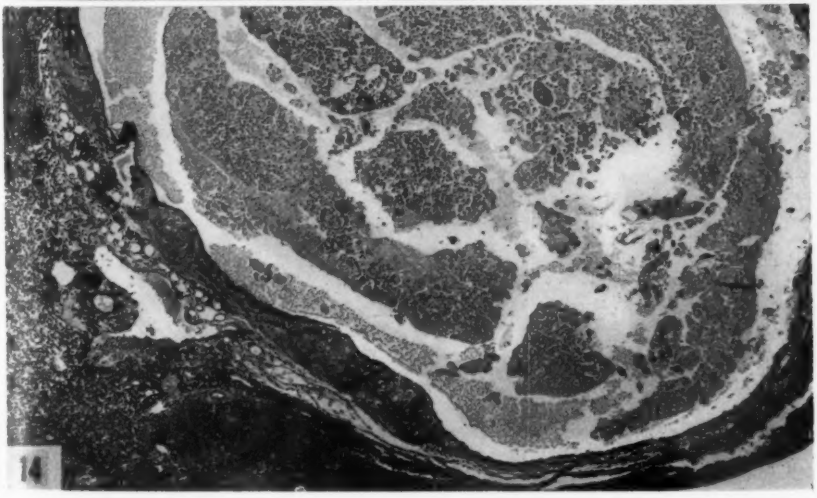
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Incidence of Leukemia and Other Tumors in Thymectomized Irradiated Mice Bearing Thymic Transplants [1, 2]

ROGER W. O'GARA *and* JEWEL ARDS,[3] *Laboratory of Pathology, National Cancer Institute,*[4] *Bethesda, Maryland*

SUMMARY

The effect of transplants of thymic tissue from newborn mice on the incidence of radiation-induced leukemia was investigated in strain C57BL mice. Thymectomized irradiated mice either were given an intrasplenic or a subcutaneous transplant of neonatal thymic tissue or were subjected to a sham intrasplenic transplantation. Mice bearing an intrasplenic transplant of thymic tissue and those given a sham intrasplenic transplant had essentially the same incidence of leukemia (10.5 and 8.7%, respectively). Mice bearing subcutaneous transplants of thymic tissue had a higher incidence of leukemia (31.6%) though not as high as the irradiated, nonthymectomized control animals (63.6%). The thymic implants were identified in the spleens of 34 percent of the animals examined during the 14-month observation period after transplantation. Histologic study of early and late implants showed normal growth and development of the thymic tissue in the spleens of irradiated mice. An apparent increase in the incidence of epithelial tumors in thymectomized X-irradiated mice is discussed briefly.—J. Nat. Cancer Inst. 27: 299-309, 1961.

THE THYMUS is involved, directly or indirectly, in the development of many spontaneous leukemias in mice and many leukemias induced by X rays or chemical carcinogens. In 1946, Furth and Boon (*1, 2*) reported that thymectomy greatly decreased the incidence of spontaneous leukemia in AK mice, and also leukemia induced by skin painting with methylcholanthrene in F_1 hybrids. Subsequently, Law and Miller demonstrated that autologous transplantation of thymus to thymectomized DBA mice restored their susceptibility to leukemia induced by percutaneous methylcholanthrene (*3, 4*). Kaplan and coworkers (*5–7*) obtained a substantial restoration of susceptibility to leukemia in thymectomized X-irradiated C57BL mice by subcutaneous transplantation of isologous adult (33-day-old) thymic tissue within 1 hour to 8 days after X irradiation. Thymic tissue from 1- to 2-day-old C57BL mice has proved more effective in

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increasing the incidence of leukemia than thymus from older animals (*8*). The susceptibility to leukemia in thymectomized irradiated mice persists for a considerable time, and lymphocytic neoplasms have been obtained in X-irradiated, thymectomized hybrid mice which received a subcutaneous transplant of thymic tissue as late as 28 days after the last dose of radiation (*9*).

Histocompatability studies have given some information on the origin of the lymphomas arising from these thymic transplants. Kaplan and coworkers concluded that the lymphoma arose from the transplanted tissue and not from the host (*10*). Law and Potter, in a similar study, confirmed the fact that some lymphomas arise from the nonirradiated transplanted thymic tissue, but found that most lymphomas arose from the irradiated cells of the host (*11*).

The growth and persistence of thymic tissue transplanted to the spleen has been demonstrated (*12*). In the present study, additional information on the relationship of the thymus to murine leukemia has been obtained by transplantation of thymic tissue from newborn mice into the spleen of thymectomized, X-irradiated adult mice.

MATERIALS AND METHODS

C57BL/6JN mice, obtained from the National Institutes of Health colony, Bethesda, Maryland, were thymectomized at about 6 weeks of age. Irradiation was begun 7 to 11 days after thymectomy, and immediately after the last dose of radiation 4 mg of streptomycin was administered subcutaneously to each mouse. After irradiation, the thymectomized animals were separated by random selection into treatment groups and received either an intrasplenic or subcutaneous transplant of thymic tissue or an intrasplenic sham transplant. The details of the procedures used in the separate experiments are given in table 1. In experiment 1, a dose of 900 r given over a period of 2 weeks killed many animals, and transplantation into the spleen was deferred until the 28th day after irradiation. In experiment 2, a 525 r dose was given, and transplantation into the spleen was made 22 to 23 days after irradiation. When it became apparent that no increase in leukemia followed intrasplenic transplantation of thymic tissue, a third experiment was done for comparison of the incidence of leukemia in animals receiving a subcutaneous transplant of thymic tissue with those receiving an intrasplenic transplant. In this experiment the dose of radiation, the interval between doses, and the time between radiation and transplantation were adjusted to conform more closely to those used by other investigators in similar studies with subcutaneous transplants (*9–11*).

The procedure for thymectomy and transplantation into the spleen has been described previously (*12*). In brief, however, one half of a thymus, which had been freshly removed from a 1- to 3-day-old C57BL mouse, was inserted into the spleen of an irradiated adult mouse by means of a

TABLE 1.—Summary of experimental procedures. Transplantation of neonatal thymic tissue into X-irradiated mice

Treatment schedule	Experiment 1 ♂ and ♀	Experiment 2 ♂ only	Experiment 3 ♂ only
Total radiation dose*	900 r	525 r	900 r
Number of divided doses	4	3	4
Interval between doses (days)	3–4	4–5	7
Number of mice irradiated	60	64	110
Number of 90-day survivors:	24	63	88
Thymectomized, intrasplenic transplant†	7	29	21
Thymectomized, intrasplenic sham transplant	6	18‡	24
Thymectomized, subcutaneous transplant†	—	—	20§
Intact (radiation only)	11	16‖	23‡
Time of transplantation (days)¶	28	22–23	7–8

*Factors: 200 kvp, 15 ma, 2 tubes, distance 34 cm from each tube, 0.25 cm Cu, 0.55 cm Al, 120.9 r per min.
†Of neonatal thymic tissue.
‡Two mice lost subsequently by cannibalism.
§One mouse lost subsequently by cannibalism.
‖Four mice lost subsequently by cannibalism.
¶Days from last radiation dose to time of transplantation.

#18-gauge trocar. The same general procedure was used for the sham transplants except that an empty trocar was used. Only mice that survived for 3 months or longer were considered in the final results.

RESULTS

A study was made of the early growth and development of intrasplenic thymic transplants in a group of irradiated mice. Twenty-nine thymectomized C57BL male mice received 525 r in 3 equal doses, and thymic tissue from newborn mice was transplanted into the spleen 7 days later. During the first 2 weeks after transplantation, 6 pairs of mice were killed at intervals of 2 to 4 days, and the remainder at weekly or monthly intervals over the next 170 days.

The grafts became visible grossly in 8 to 10 days as large, white nodules beneath the splenic capsule (fig. 1). Histologic study showed that the grafted thymus became repopulated from surviving thymocytes in 4 to 6 days after transplantation (figs. 2 and 3). The thymocytes in the intrasplenic graft closely resembled those of similar grafts previously studied in nonirradiated mice (*12*), and no differences in reaction or morphologic appearance were noted that could be attributed to the effects of prior irradiation.

Other groups of animals were allowed to live 14 months after irradiation, unless they died or became moribund sooner (tables 1 and 2), and the transplanted portion of thymus was identified in the spleens of 34 percent of these animals. Microscopically, the transplanted thymic tissue formed a discrete mass distinct from the lymphoid tissue of the spleen (fig. 4). Frequently the transplanted thymus had a distinct cortex and medulla, and groups of fat cells were sometimes found within the grafted tissue. The cells were more closely packed in the thymic

TABLE 2.—Number of lymphocytic neoplasms in C57BL/6JN mice after irradiation

Treatment	Total number irradiated	Months after irradiation											Total	Total dead from other causes	Survivors (14 months)
		2–3	4	5	6	7	8	9	10	11	12–13	14			
Expt. 1 (900 r)															
Thymectomized															
Intrasplenic graft*	7	—	—	—	—	—	—	—	—	—	—	—	0	1	6
Sham intrasplenic graft	6	—	—	—	—	—	—	1	—	—	—	—	1	1	4
Intact (radiation only)	11	—	2	1	1	3	—	—	—	—	—	—	7	2	2
Expt. 2 (525 r)															
Thymectomized															
Intrasplenic graft*	29	—	—	—	—	—	2	—	—	—	—	1	3	4	23†
Sham intrasplenic graft	16	—	—	—	—	—	—	1	—	—	—	—	1	1	14
Intact (radiation only)	12	—	2	2	1	—	—	1	—	—	—	—	6	0	6
Expt. 3 (900 r)															
Thymectomized															
Intrasplenic graft*	21	—	—	—	1	—	—	1	—	1	—	—	3	3	15
Sham intrasplenic graft	24	—	—	1	—	—	—	—	1	—	—	—	2	7	15
Subcutaneous graft*	19	—	1	2	—	—	—	1	—	1	—	1	6	2	12†
Intact (radiation only)	21	—	3	3	3	3	—	3	—	—	—	—	15	2	4
Total (all experiments)															
Thymectomized															
Intrasplenic graft	57	—	—	—	1	—	2	1	—	1	—	1	6(10.5%)	8	44†
Sham intrasplenic graft	46	—	—	1	—	—	—	2	1	—	—	—	4(8.7%)	9	33
Subcutaneous graft	19	—	1	2	—	—	—	1	—	1	—	1	6(31.6%)	2	12†
Intact (radiation only)	44	—	7	6	5	6	—	4	—	—	—	—	28(63.6%)	4	12

*Of neonatal thymic tissue.

†Including one mouse with leukemia at 14 months post irradiation.

grafts, and the nuclei of the thymocytes were more hyperchromatic, stained more sharply, and were more regular in size and shape than the nuclei of lymphocytes in the follicles of the surrounding spleen (figs. 5 and 6).

Subcutaneous grafts were positively identified in only 3 of 19 animals. Of these, 2 had generalized leukemia, but the thymic graft was involved in only one. In a fourth, a large subcutaneous lymphosarcoma arose at the site of the graft and obliterated it.

The number of mice that developed lymphocytic neoplasms during a 14-month observation period after irradiation is shown in table 2. Mice still living 14 months after irradiation were killed, and 2 additional lymphocytic neoplasms were found at autopsy. Thymectomy decreased the incidence of lymphocytic neoplasms induced by irradiation from 64 percent in intact control mice to 9 percent in sham-transplanted, thymectomized control mice ($P<0.001$). There was an apparent partial restoration (32% incidence) in susceptibility to leukemia in thymectomized animals bearing subcutaneous implants of thymic tissue ($P = 0.1$), but no increase in susceptibility (10% incidence) in thymectomized animals bearing intrasplenic thymic grafts.

Neoplasms were considered to be of thymic origin if they involved (grossly or microscopically) either the site of a thymic graft, the thymus of intact animals, or the anterior mediastinum of thymectomized animals. In the latter, the tumors were regarded as having arisen in remnants of thymus possibly left behind at thymectomy. The origins of the induced lymphocytic neoplasms have been tabulated in table 3. The incidence of tumors of thymic origin was 50 percent in the intact mice and 2 to 4 percent in thymectomized mice bearing an intrasplenic thymic or a sham transplant. The incidence of lymphocytic tumors arising elsewhere than in the thymus was 14 percent in the intact mice and 7 percent in the

TABLE 3.—Lymphocytic neoplasms of thymic and nonthymic origin in irradiated C57BL/6JN mice

Group	Total number of mice	Lymphocytic neoplasms							
		Total number	Percent	Thymic origin*			Nonthymic origin†		
				Number	Percent	Average age (months)	Number	Percent	Average age (months)
Thymectomized:									
Intrasplenic graft	57	6	11	2‡	4	7.5	4	7	10.2
Sham intrasplenic graft	46	4	9	1‡	2	9.0	3	7	8.0
Subcutaneous graft	19	6	32	3§	16	7.7	3	16	8.3
Intact (radiation only)	44	28	64	22	50	6.2	6	14	5.0

*Involving the thymus, thymic region, or thymic graft.
†Not involving the thymus, thymic region, or thymic graft.
‡Involving the thymus region (residual thymus?).
§One tumor involved the thymic region (residual thymus?) and two involved the graft site.

thymectomized mice bearing either an intrasplenic thymic or a sham transplant. Thus, an intrasplenic thymic graft had no demonstrable influence on the incidence of leukemia in thymectomized X-irradiated mice. On the other hand, a subcutaneous graft gave an apparent increase in the incidence of leukemia arising from both thymic and other lymphoid tissue, but a larger group of animals would be required to establish the statistical significance of this difference.

Table 4 gives the incidence of neoplasms, other than lymphocytic neoplasms, which arose in X-irradiated mice. Only mice autopsied 12 to 16 months after irradiation are included in this tabulation, to minimize the bias due to the high incidence of leukemia in the intact animals. It is apparent from the table that a large variety of neoplasms arose in X-irradiated mice that survived for a sufficiently long time after irradiation. All these neoplasms arose in thymectomized animals.

TABLE 4.—Nonlymphocytic neoplasms in C57BL/6JN mice autopsied 12 to 16 months after irradiation

Neoplasms	Thymectomized			Intact (radiation only)
	Intrasplenic thymic graft	Intrasplenic sham graft	Subcutaneous thymic graft	
Benign polyp of stomach	1	0	1	0
Benign polyp of colon	4	2*	0	0
Hepatoma	1†	2	0	0
Lung adenoma	2†	1	0	0
Mammary carcinoma‡	1†	0	0	0
Fibrosarcoma of testis	0	1*	0	0
Lymphangioma	1†	0	0	0
Interstitial-cell adenoma	1	0	0	0
Granulosa-cell carcinoma§	1	0	0	0
Reticulum-cell sarcoma	1	3	0	0
Parathyroid adenoma	1	0	0	0
Total number of tumors	14	9	1	0
Total mice with tumors	*11*	*8*	*1*	*0*
Total mice in group	50	39	14	12
Percent of mice with tumors	22.0	20.5	7.1	0.0

*One animal had 2 tumors.
†One animal had 4 tumors.
‡Found in male animal.
§Found in female animal.

DISCUSSION

The results of these experiments make it apparent that the transplantation of thymic tissue into the spleen of thymectomized X-irradiated C57BL mice does not increase the incidence of radiation-induced leukemia. Since subcutaneous transplantation of thymic tissue under these conditions results in a considerable increase in the incidence of leukemia (*6–11*), there appears to be a fundamental difference between subcutaneous and intrasplenic transplantation of thymic tissue. Comparative studies on the growth of thymic transplants have shown that implants in spleen regenerate faster than those in the subcutaneous tissue (*12*). It is possible that, because of its more protracted period of degeneration, the subcutaneous thymic transplant is more susceptible to leukemogenic in-

fluences of the irradiated host. A second possibility is that, although the thymic implant grows better in the spleen, any leukemogenic influence that it may exert is removed from the portal blood during its passage through the liver. The first possibility seems the more likely one, since well-developed transplants of thymic tissues in the kidney of thymectomized irradiated C57BL mice do not increase the incidence of leukemia in thymectomized irradiated mice (*13*).

The incidence and variety of neoplasms of epithelial and other nonlymphocytic origin is striking and warrants comment. All these tumors, which were found 12 to 16 months after irradiation, occurred in thymectomized animals. This suggests that thymectomy, while decreasing the incidence of leukemia, may render the animal more susceptible to nonleukemic carcinogenesis. Since only a few intact irradiated animals survived for 12 months, an alternate possibility is that the animals in this group most sensitive to carcinogenesis died early from leukemia instead of surviving to develop other tumors. Furth (*2*), using hybrid mice which were relatively resistant to leukemia induction from methylcholanthrene skin painting (35% leukemia in 12 months), reported a considerably higher incidence of tumors of the skin, breast, bone, and lung in thymectomized mice as compared to intact mice. Furth interpreted this higher incidence of nonlymphocytic neoplasms in thymectomized mice as probably due to earlier deaths of the intact animals from leukemia. It has also been noted (*12*) that in old nonirradiated mice (21–24 months), a higher incidence of spontaneous nonlymphocytic neoplasms was obtained in thymectomized than intact animals.

In the present experiments, thymectomized mice bearing subcutaneous thymic transplants, like the intact animals, developed very few epithelial tumors. Since these animals are part of experiment 3 (900 r) in which most of the epithelial tumors were found, a further difference is suggested in the character of the subcutaneous versus the intrasplenic thymic transplants. Only further work with larger groups of animals can determine whether this influence of thymectomy on the incidence of epithelial tumors is real or simply apparent.

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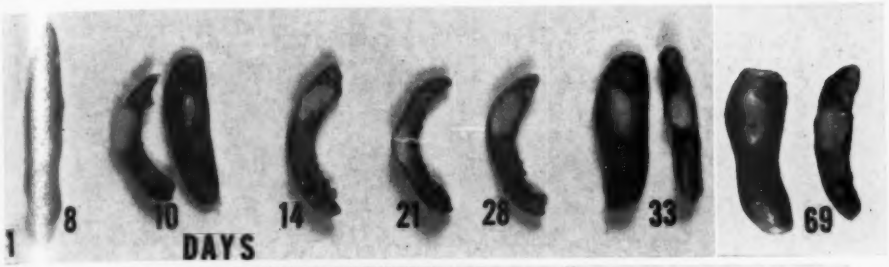
PLATE 37

FIGURE 1.—Gross photographs of transplanted thymic tissue in spleens of thymectomized X-irradiated mice 8, 10, 14, 21, 28, 33, and 69 days after transplantation. × 2

FIGURE 2.—Microscopic appearance of thymic tissue 2 days after transplantation into spleen of thymectomized X-irradiated mouse. Darkly staining cells just beneath splenic capsule are thymocytes. Splenic tissue is present in *lower* portion of photograph. One lymphoid follicle of spleen is visible at *left*. Hematoxylin and eosin. × 80

FIGURE 3.—Thymic tissue 4 days after transplantation into spleen of thymectomized X-irradiated mouse. Thymic tissue is just beneath splenic capsule and is distinct from adjacent splenic tissue. Hematoxylin and eosin. × 75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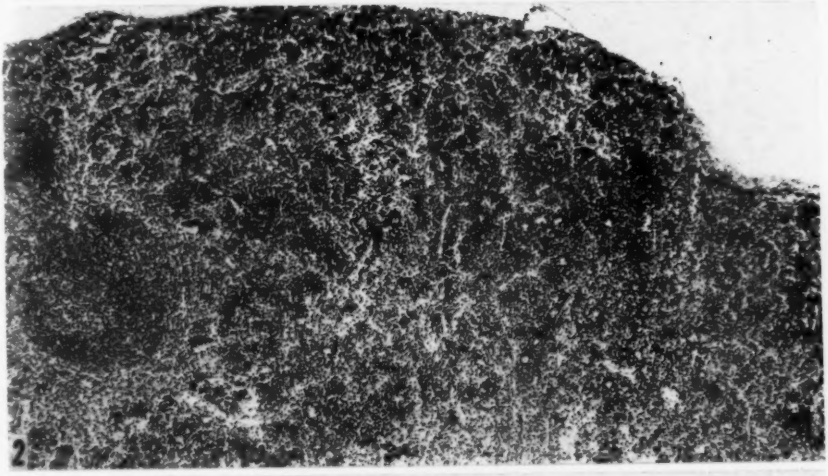
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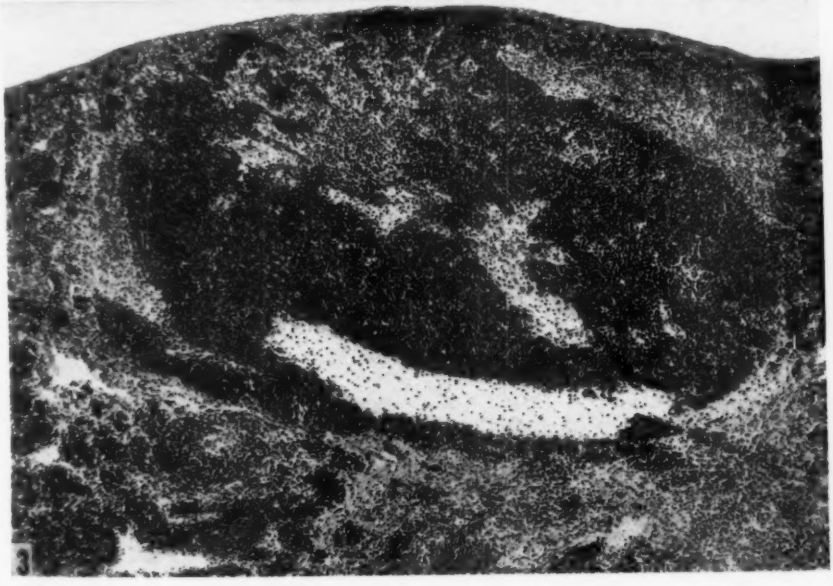
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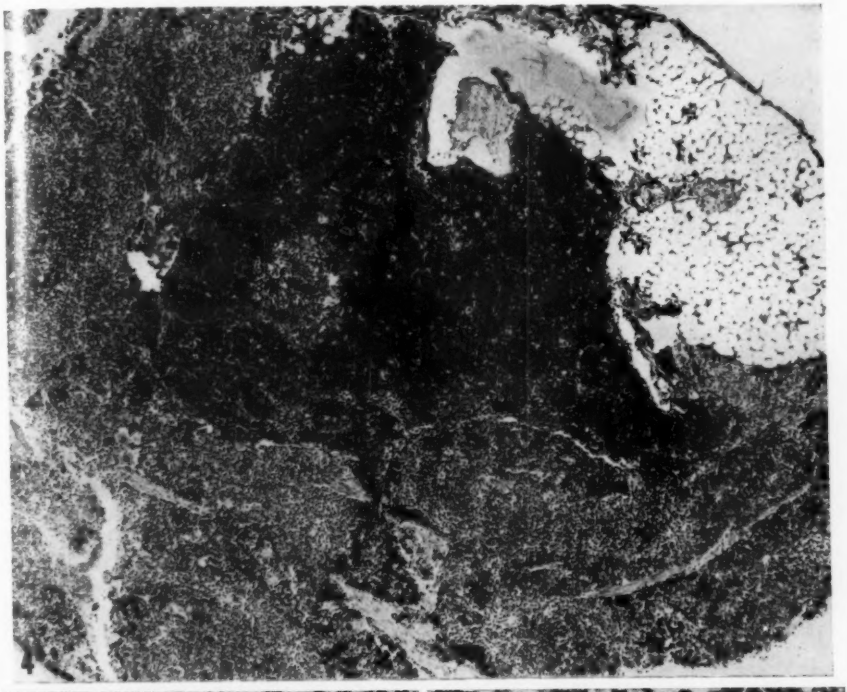
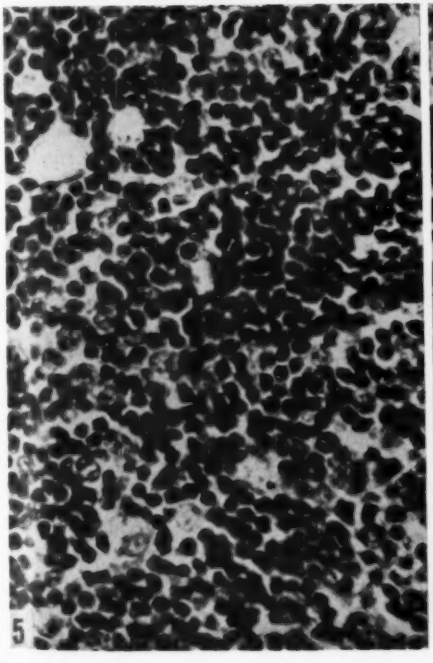
PLATE 38

FIGURE 4.—Thymic graft 14 months after transplantation into spleen of thymectomized, X-irradiated mouse. Darkly staining thymocytes form circumscribed nodule within spleen. Between graft and splenic capsule (*upper right* corner), adipose tissue has developed from partial involution of thymic graft. Small finger of epitheloid cells projects outward from graft into adipose tissue. Hematoxylin and eosin. × 75

FIGURE 5.—Higher magnification of grafted thymic tissue in figure 4. Thymocytes are closely packed and hyperchromatic but relatively uniform in size and appearance. Hematoxylin and eosin. × 610

FIGURE 6.—Higher magnification of lymphoid nodule from spleen in figure 4. *Compare* with figure 5. Hematoxylin and eosin. × 610





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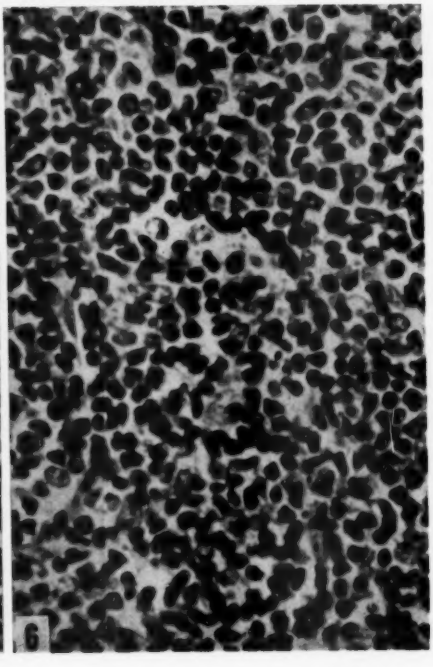

6

Cytophotometric Data on Fluorochromed Cervical Cells [1,2]

MARIANNA MASIN, *M.D.*, *and* FRANCIS MASIN, *M.D.*,[3,4] *Research Department, Mount Sinai Hospital, Los Angeles, California*

SUMMARY

A simplified fluorophotometric device for measuring the light emission of the whole-cell area of single isolated cells is described. Ehrlich ascites carcinoma cells stained with the basic fluorochrome acridine orange were used for a model experiment. The sensitivity of the used photomultiplier 1P21 for the green-yellow emitted by the nuclear area was about 4 times greater than that for the metachromatic red of the cytoplasmic and nucleolar ribonucleic acid. Galvanometer readings over concentric cell areas of varying diameters showed that nuclear emission was mainly responsible for the total recorded fluorescence intensity. Photometric data obtained on single fluorochromed cervical cells gave mean emission values approximately 2 times higher for abnormal than for corresponding normal cells. Emission values of abnormal cells were distinctly above the level of a dividing line at 36.2 emission units and were well separated from normal cells. The differential staining of nucleus and cytoplasm by a basic fluorochrome with well-delineated nucleo-cytoplasmic boundaries is of advantage for detection of cellular atypia by visual observation. In addition, cytophotometric data can yield valuable evidence in closer identification and separation of degrees of cellular abnormalities, especially where cytologic classification and tissue findings are not conclusive.—J. Nat. Cancer Inst. 27: 311-331, 1961.

THE PURPOSE of this study was to evaluate the usefulness of a simple cytophotometric instrument as a supplementary device to routine cytologic screening in instances where the cytologic classification presents diagnostic problems. A measure of the light emitted by fluorochromed cervical cells could contribute additional information about the varying degrees of cellular abnormalities and clarify identification of elements consistent with epithelial malignancy. Furthermore, in view of a mechanized screening device, the obtained measurements could give

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[3] *Present address*: Analytical Cytology Research Laboratory, The Hospital of the Good Samaritan, 1212 Shatto St., Los Angeles 17, Calif.

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objective norms for sharper distinction between normal and abnormal cells and eventually be helpful in the establishment of more clearly defined categories for different types of cellular atypias.

In previous microfluorometric studies (*1–3*) a basic fluorochrome berberine sulphate was used to stain the nuclei for differential detection of neoplastic cells. In the present study, the data were obtained from cervical smears fluorochromed by acridine orange (AO), by a staining method previously described for gynecological materials (*4, 5*). This method allows for differential staining of ribonucleic (RNA) and deoxyribonucleic acid (DNA), and the growth gradient in the maturing squamous epithelium of the cervix and vagina is evidenced by the decreasing content of RNA toward the surface layers (maturation and loss of basophilia). However, the AO fluorochrome, like other basic dyes, is not bound exclusively by the phosphate groups of nucleoproteins; other acidic groups and factors could contribute to its nonspecific binding (*6*). Increased cytoplasmic basophilia *per se* is an expression of rapid cell growth, and evidently cannot be the sole factor when the abnormal character of cells is judged. Its presence or increase depends also on the particular tissue type under study. In the squamous-cell carcinomas of the uterine cervix (*7*) there are many cells with acidophilic cytoplasm (30.9%), and the proportion of the basophilic and acidophilic cells depends on the degree of anaplasia of the particular neoplasm. Well-preserved proliferating, transitional, and degenerating cells are present in every cervical cell population and exhibit variable stages in their preservation. Consequently, cellular elements of the same type show a varying degree of cytoplasmic basophilia and acidophilia, as well as a varying concentration of nuclear chromatin. By the method of cervical scraping, the nuclei are damaged less easily than the cytoplasm, and their chromatin is well preserved as shown by staining. In degenerating tumor cells the labile cytoplasmic RNA frequently shows more advanced depolymerization than the relatively stabile DNA (*8*). The fluorescence of AO bound to the cellular structures covers the spectral range from green through yellow to red (*9, 10*). The degree of cytoplasmic basophilia and nuclear hyperchromasia of cervical cells stained by the AO fluorochrome (in red-orange and green-yellow, respectively) indicates different concentrations of the acidic groups of nucleoproteins available as binding sites. In contrast, the acidophilic cytoplasm stains in varying intensities of green, representing cell proteins stained below their isoelectric point (*11*).

In this study, while selecting single fluorochromed cervical cells for measurement, we recorded a detailed description of cellular morphology, varying color intensities, cell preservation, and general appearance of smears. These data were collected for comparison between cytophotometric values, cellular atypical features, AO classification (*12*), and epithelial abnormalities found in tissues.

Rapidly growing abnormal cells with high concentrations of RNA and DNA, after fixation and staining with the fluorochrome AO, frequently exhibit similar increased intensities of red-orange in the cytoplasm and

yellow-green in the nuclei. Fine differences in color intensities are difficult to estimate visually, and in spite of the indisputable excellence of cytoplasmic and nuclear structural details brought out by fluorescence microscopy and the degree of cellular basophilia evidenced by cationic dye staining, appreciation of the degree of abnormality in such elements is often very difficult (*13*). Therefore, we obtained fluorescence emission data on cells by a microfluorometric device to establish a procedure for more objective detection of the varying stages of cellular abnormalities. Some of these are difficult to identify with the current cytologic methods, but their relationship to the respective tissue pattern could be evidenced by such photometric data.

The fluorescence of the whole cell represented emission from a heterogeneous area comprising all structures of the cytoplasm and nucleus, and was recorded for detection of relative emission differences between cells, which, expressed in arbitrary units, could be useful in differential cytologic classification. For information on the proportional participation of the nuclear and cytoplasmic fluorescence in the total light emission, data on AO fluorochromed Ehrlich ascites carcinoma cells were collected in a model experiment.

PART 1. STUDY OF FLUOROCHROMED EHRLICH ASCITES CARCINOMA CELLS

Materials and Methods

Ehrlich ascites carcinoma cells, grown for 7 days in a Swiss strain mouse, were aspirated, carefully spread on slides for acquisition of undamaged cells, fixed in alcohol-ether (1:1), and stained by the acridine-orange procedure (*5*). These smears yielded a morphologically uniform cell population suitable for measurements. A microfluorophotometric device was assembled from commercially available components (Leitz) [6] according to principles described by Pollister (*14*).

A Leitz research microscope (Ortholux) was combined with a sliding attachment equipped with an iris, a detector 1P21 phototube, a power supply, and a Rubicon galvanometer with a sensitivity of 0.0010 μamp/mm. A voltage stabilizer was inserted into the circuit. A centerable fieldstop was installed in the microscope as well as an achromatic, aplanatic condenser for optimum illumination of the specimen. An apochromat objective (40×) was furnished with a funnel stop for glare control. The object was magnified (160×) at the image screen, and the alignment of the whole unit was checked by a telescopic eyepiece. Test runs were performed and calibration curves recorded. The diaphragm of the sliding attachment, which controlled the field size projected on the phototube surface, was adjustable from an opening of 1 through 20 mm. We used a 5 mm diaphragm opening since this was the width of the sensitive grid of the phototube. This aperture, corresponding to 31 μ

[6] We wish to acknowledge the helpful advice of Mr. Herman Adler, from the Scientific Instrument Division of the E. Leitz, Inc., New York City.

on the object level, satisfactorily covered the average diameter of carcinoma cells (*15–17*). A 30W condensed tungsten filament bulb was used as a source of exciting light, which produced a brilliant fluorescence and was cold enough to avoid fading of the fluorochromed cells during measurements. This blue-violet light was obtained by combination of 2 Corning filters with a maximum transmittance of 400 to 450 and 340 to 660 mμ. The yellow-barrier filter secured a steep cutoff at 510 mμ. This crossed filter combination gave a satisfactory black background. We standardized the intensity of the exciting beam by passing it through filters placed on the level of the fieldstop and checking the response elicited on the galvanometer with a constant sensitivity setting. The sensitivity of the phototube 1P21 shows the peak at 4,000 A. However, the inserted yellow filter excludes the violet-blue exciting radiation, preserving phototube sensitivity at 5,500 A (green region) about 4 × higher than at 6,000 A (orange-red portion).

Procedure for Measuring Cell Fluorescence

An isolated cell was moved to the center of the field (so that no neighboring cells which could contribute any interfering fluorescence were present in the measured field), brought into sharp focus, and the iris closed to the 5 mm opening. If the projected image of the cell was larger than the opening of the iris, only the area defined by the iris with the nucleus centered therein was measured. Only cells which had perfectly centered nuclei were selected for measurement. We took great care to select well-preserved cells appearing undamaged and not degenerated with seemingly intact cellular borders.

In a preliminary study, data on 10 cells were collected by a first measurement of their fluorescence with an iris opened to 5 mm, then by a second after the diaphragm was narrowed so as to encircle as nearly as possible the outer rim of the cytoplasm, and thus, the whole cell area. The third measurement encompassed only the nucleus (table 1).

TABLE 1.—Measurements on fluorochromed Ehrlich ascites carcinoma cells, with maximum useful opening of the diaphragm 5 mm

	Diaphragm at rim of cytoplasm		Diaphragm at rim of nucleus	
Galvanometer reading	Opening (mm)	Galvanometer reading	Opening (mm)	Galvanometer reading
80	4	68. 5	3. 5	40
65	3	50	3. 5	38
71	4	61	3. 0	45
25	2. 5	19	1. 5	13
23	—	—	—	—
35	—	—	—	—
55	3. 5	45	2. 5	31
53	3	39	—	—
57	4	50	3	36
31	2. 75	26	2	20

A second series of 59 measurements on fluorochromed Ehrlich ascites carcinoma cells was performed while the opening of the diaphragm was gradually decreased (5, 4, 3, and 2 mm) regardless of the boundaries of the cytoplasm and nucleus. Participation of the cell areas responsible for the measured emission at different diaphragm openings was as follows: At the 5 mm opening (diameter at the image plane, corresponding to 31 μ on the specimen level), the whole cell was usually encompassed. Larger elements were not measured, as the readings would be off the galvanometer scale. At the 4 mm opening, a portion of the cytoplasm and the whole nucleus were included in larger cells, whereas in smaller cells the whole cell area was included. At 3 mm (approximately 18 μ), usually the entire nucleus was encompassed. At 2 mm, either the entire nucleus or only a part of the inner nuclear area was included. Measurements of the background were performed at the same openings to determine if the transmitted light passing through the cross-filter combination would influence the cell measurements.

Results for Part 1

Distribution of Ehrlich ascites carcinoma cells into different classes according to the fluorescence emitted from the whole cell area is given in tables 2 and 3. Analysis of fluorescence emission values on these single cells (table 3) showed the following: Emission readings were plotted by groups according to the emission at different diaphragm openings (text-fig. 1). The curves appeared to be very similar except that there was comparatively greater uniformity in light emission at the 2 mm opening. There was some evidence that the readings for cells with an emission of 80 galvanometer units or more were about equal for the 2 mm opening as for the 5 mm opening, while the emission readings of the other groups dropped to a greater extent when the 5 mm readings moved toward 40.

TABLE 2.—Distribution of emission values of fluorochromed Ehrlich ascites carcinoma cells according to classes

Range of galvanometer readings	Number of cells
120–110	3
110–100	8
100–90	8
90–80	14
80–70	9
70–60	11
60–50	4
50–40	2
	Total 59

TABLE 3.—Emission readings of AO fluorochromed Ehrlich ascites carcinoma cells at different diaphragm openings

Galvanometer reading	Number of cells	Opening (mm)			
		5	4	3	2
110–119		112	108	95	65
		111	104	82	51
		110	99	72	45
Mean	3	111. 0	103. 7	83. 0	53. 7
100–109		103	98	79	41
		104	102. 5	87. 5	38
		100	97	86	48
		101	98	80	54
		100	94	71	—
		105	103	70	45
		104	95	76	49
		100	98	82	50
Mean	8	102. 1	98. 2	78. 9	46. 4
90–99		98	92	76	53
		93	90	63	28
		93	90	75. 5	45
		91	88	73	44
		90	87	79	53
		98	93	76	48
		98	96	84	47
		99	96. 5	82	52
Mean	8	95. 0	92. 5	76. 0	46. 2
80–89		86	83	64	42
		80	78	73	47
		88	86. 5	69	44
		82	80	79	46
		84	82	69	44
		81	78	68. 5	47. 5
		83	81	68	45
		80	78	73	52
		87	83	65	42
		80	78	64	29
		82	79	68	45
		82	79	69	47
		83	79	64	37
		83	80	75	54
Mean	14	82. 9	80. 3	69. 2	44. 2
70–79		72	69	61	40
		78	76	70	46
		74	70	60	43
		75	72. 5	64	45
		76	74	64	39
		74	73	65	45
		78	75	69	40
		79	76	66	44
		79	73	66	44
Mean	9	76. 1	73. 1	65. 0	42. 9
60–69		64	62. 5	59	43. 5
		61	59. 5	55	35
		61	58	54	40
		69	63	43	—
		63	61. 5	58	41
		65	63	61	43
		66	64	56	31
		69	67	57	42
		63	62	49	35
		69	67	60	45
		69	66. 5	54	35
Mean	11	65. 4	63. 1	55. 1	39. 0

TABLE 3.—Emission readings of AO fluorochromed Ehrlich ascites carcinoma cells at different diaphragm openings—Continued

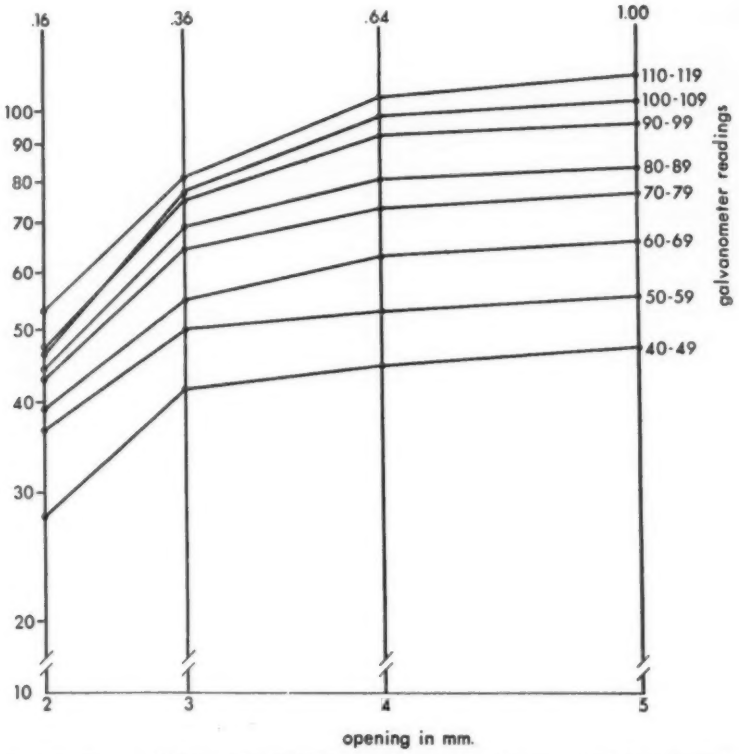
Galvanometer reading	Number of cells	Opening (mm)			
		5	4	3	2
50–59		57	55	50	35
		57	55	52	38
		55	53	51	37
		51	49	47	37
Mean	4	55. 0	53. 0	50. 0	36. 8
40–49		45	42	40	30
		49	47	43	26
Mean	2	47. 0	45. 0	41. 5	28. 0
For three groups	Number of cells	Opening (mm)			
		5	4	3	2
>80	33				
Mean		93. 0	89. 7	74. 4	46. 1*
60–80	20				
Mean		70. 2	67. 6	59. 6	40. 8*
<60	6				
Mean		52. 3	50. 2	47. 2	33. 8

*Mean based on one less case.

In text-figure 2, the results were plotted to study the same phenomenon. It showed the emission readings at the 5 mm opening plotted against the readings at the 2 mm opening. On the basis of this graph, we decided to analyze the data separately for different readings at the 5 mm opening. They were subdivided into three groups: readings greater than 80, between 60 and 80, and less than 60. These are shown in table 3 together with mean emission readings for each opening, with cells divided into groups by intervals of 10.

An impression of the variation in fluorescence emitted by different areas of the cell can be obtained from a comparison of the readings for each of the different size openings. This analysis was carried out for the 3 groups mentioned (table 3). The openings used were circles of diameters 2, 3, 4, and 5 mm opening corresponding to 31 μ diameter field size. The circles have areas in proportion to their squares: 4, 9, 16, and 25. The annular rings have areas in proportion to 1.00, 1.25, 1.75, and 2.25.

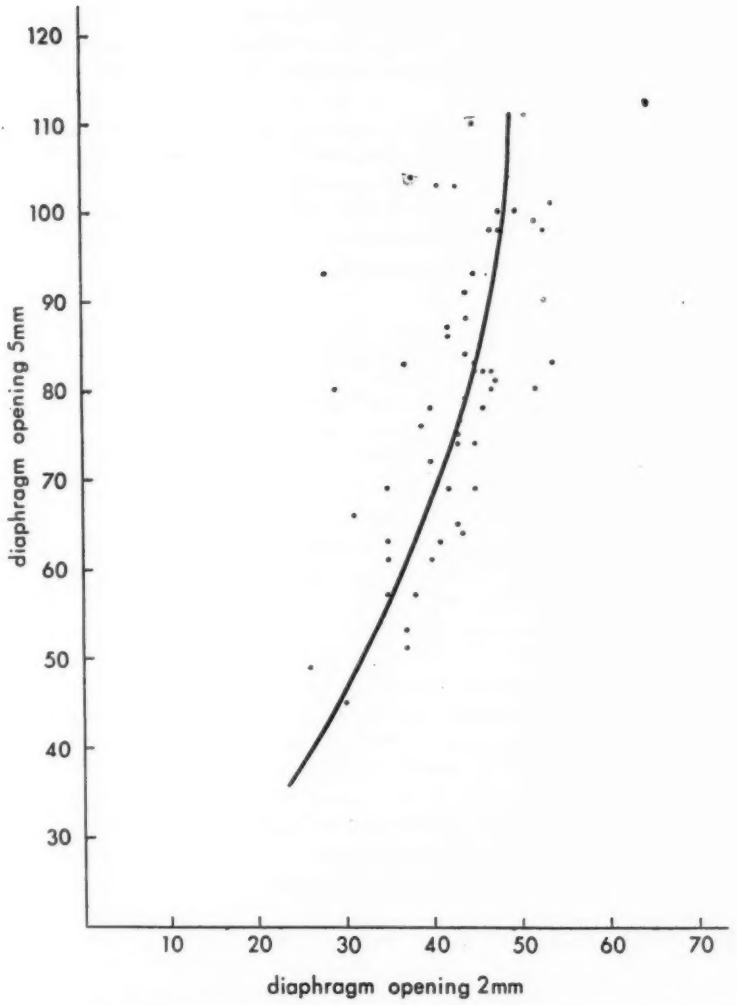
Table 4 presents the mean light emission readings on Ehrlich ascites carcinoma cells. Part A gives the average emission readings over the annular rings, while part B lists the mean values for unit emission at the center. This part shows the decreasing light emission in each ring as a proportion of the light emitted at the center. Part C shows the results of an additional adjustment based on the areas of the rings. The resultant



TEXT-FIGURE 1.—Mean light-emission readings at different diaphragm openings. Values were extracted from table 3.

Table 4.—Mean light-emission readings on Ehrlich ascites carcinoma cells

	For 3 groups at 5 mm diaphragm opening	Ring			Center
		4–5 mm	3–4 mm	2–3 mm	2 mm
A	>80	3. 3	15. 3	28. 4	46. 1
	60–80	2. 6	8. 0	18. 8	40. 8
Over annular rings	<60	2. 2	3. 0	13. 3	33. 8
B	>80	0. 072	0. 331	0. 616	1. 0
	60–80	0. 064	0. 197	0. 460	1. 0
For unit emission at center	<60	0. 064	0. 089	0. 394	1. 0
C	>80	0. 032	0. 189	0. 493	1. 0
	60–80	0. 028	0. 113	0. 368	1. 0
For unit emission at center adjusted for size of annular ring	<60	0. 028	0. 051	0. 316	1. 0
D	>80	1. 5	8. 7	22. 7	46. 1
	60–80	1. 2	4. 6	15. 0	40. 8
Adjusted for area of annular ring—center taken as unit ring	<60	1. 0	1. 7	10. 6	33. 8
E	>80	12	72	187	381
	60–80	10	38	124	337
Per 1,000 μ^2	<60	8	14	89	279



TEXT-FIGURE 2.—Light-emission readings at the 5 mm diaphragm opening plotted against readings at the 2 mm opening. Values were extracted from table 3.

figures are now proportional to the emission per unit area. The emission decreases through all rings, but differently for each group of galvanometer readings, and attains about the same level at the outside ring. Part D preserves the emission readings as obtained for the center circles and adjusts the other readings on the basis of equal area. These figures were estimated emissions per unit area at various distances from the center. Some of the increased emissions obtained from cells of greatest over-all light emission arise from the portions of the cell in rings with openings up

to 3 and 4 mm at the image level. Since the area of the center circle with a 2 mm opening corresponds to the area $\frac{1}{4}\ (\frac{2}{3})^2\pi 31^2 = 121\mu^2$, the emission values per 1,000 μ^2 can be computed from part D. These values are given in part E.

Analysis of data on single fluorochromed Ehrlich ascites carcinoma cells showed that, with the described detector unit, the green-yellow fluorescence of the nuclear area was the main contributing factor in the total light emission. The readings over the central cell area were at least 10 times higher than values recorded over the cell periphery, which contained cytoplasmic RNA fluorescing in red (table 4).

PART 2. CYTOPHOTOMETRIC DATA ON SINGLE CERVICAL CELLS

Cervical smears from normal, abnormal, and malignant epithelia were prepared from patients. To obtain relatively "fresh" cellular elements that had not been exposed to excessive enzymatic degradation as occurs in vaginal smears, we prepared them by the scraping technique. Fixation, staining procedure, and instrumental set-up used were the same as described in Part 1. Single, well-preserved, and transitional cells of normal and abnormal cell types were included in measurements to encompass the whole range of a variable cell population. Malignant, naked nuclei were also measured, but completely degenerated cells were excluded. Such elements with extensive damage to cytoplasmic or nuclear surfaces and with profound alteration of their chemical components naturally have altered staining characteristics (*13*) and consequently are not suitable for cytophotometric measurements.

Since it is not possible to obtain exclusively well-preserved cells by the routine smear-preparation techniques, such broad sampling was chosen in view of a potential use of cytophotometric measurements as a supplementary device for sharper identification of abnormal cells in routine cytologic smears, or for use with a mechanized screening device. For measurements, we selected only single, well-isolated cells to avoid interference from the glare of other surrounding elements, especially from nuclei of leukocytes.[6] No attempt was made in this part of the study to use special cell-collection or cell-distribution techniques, since the primary purpose was to test the suitability of routinely prepared smears for collection of cytophotometric data. However, in pilot experiments, a modified single-drop technique (*18*) was used satisfactorily in combination with a vibrating stage (*19*) to obtain an improved cell distribution.

Analysis of Results

Table 5 tabulates the results of a survey of 819 cells measured in cervical smears of all AO classes. For diagnostic evaluation of cervical smears the AO categories I through V were used, as reported in a previous study

[6] The competent technical assistance of Mrs. Joan Maudlin, cytotechnologist, is gratefully acknowledged.

(*12*). The classification is based on a combination of morphologic features and/or increase in cellular basophilia, as established by observation of fluorescence colors in normal and abnormal cells (*5*, *12*). The measured elements were distributed into 2 large groups of "normal" and "abnormal" cells (tables 5 and 8). The normal group included all epithelial cell types represented in smears obtained by scraping of the exocervix, the youngest inner basal cells, and the most mature cornified cells. In the abnormal group (tables 5 and 8) "atypical" cells from smears of AO classes III, IV, and V were included, which represented elements with marked morphologic and/or abnormal fluorescence characteristics. These formed a relatively large group in AO class III, due to the already previously reported observation (*12*) that more atypical cells are detected by the AO method than by the Papanicolaou technique. "Suspicious" and "malignant" cells represented those with increasing degrees of abnormality (as characterized by the AO method) and were largely found in smears of AO classes IV and V. Malignant, naked nuclei were also included in the abnormal group, because of their frequent presence in smears from malignant epithelia and their significance in cytologic classification.

To establish mean light-emission values for cells from normal cervical epithelia, we prepared smears from 10 patients (of AO classes I and II), and performed cell measurements on all cell types encountered, as tabulated in table 6. These cell types included cornified, precornified, intermediate, outer, and inner basal cells, and showed that the light-emission reading increased from the cornified cells to the inner basal cells, and reflected the increasing growth gradient or degree of immaturity of the younger elements. These values obtained on normal cells were used for comparison with data obtained from smears of AO classes III, IV, and V, as outlined in table 8. Variations in values obtained for normal cell types in 10 patients were most probably due to individual variations as well as to differences in age, hormonal status, and varying thickness of the cervical epithelium.

As evident from table 6, all cells gave light-emission values that were less than 35 units. These mean values resulted from the observation of from 1 to 13 cells of a single-cell type for each patient. Altogether, 274 cells were measured.

Text-figure 3 illustrates the data obtained on normal cells from smears of AO classes I and II. The solid connecting line represents the mean of the mean emission values for each particular cell type across the 10 patients, and falls well within the "critical light-emission" line (at 36.2 units) of galvanometer reading. There is a uniformity of emission readings across the cell types for different patients with cervical smears of AO classes I and II. A partial analysis of variance was performed for the cornified, precornified, and intermediate cell types (table 7). Such analysis would also be similar for the outer and inner basal cells. However, these cell types could not be included in the analysis, since not all patients had such less mature cell types represented in their cervical smears.

TABLE 5.—Survey of measured cell types in different AO classes*

AO class	Number of patients	Normal cell types							Abnormal cell types†					Total of all cells in class
		Cornified	Precornified	Intermediate	Outer basal	Inner basal	Naked nuclei	Total	Atypical	Suspicious	Malignant	Malignant, naked nuclei	Total	
I & II	11	59	101	69	43	23	1	296						296
III	8	21	41	33	30	11	3	139	9	5		1	15	154
IV	8	17	24	36	44	26	5	152	7	3		6	16	168
V	6	30	35	30	15	13	8	131	3	27	31	9	70	201
Total	33	127	201	168	132	73	17	718	19	35	31	16	101	819

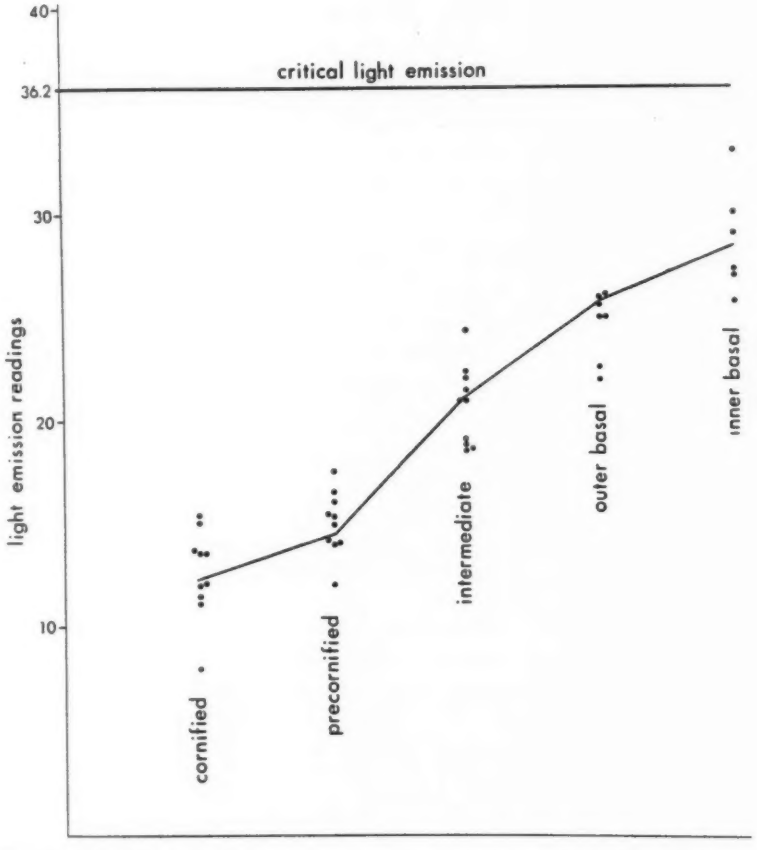
*Numbers represent total of single cell measurements.
†For description of abnormal cell types, *see* "Analysis of Results."

TABLE 6.—Mean light-emission readings of different cell types in cervical smears (AO I and II) from 10 patients*

Cell type	1	2	3	4	5	6	7	8	9	10	Mean of mean	SD
Cornified	11. 2(10)	12. 2(5)	12. 0(2)	13. 7(7)	13. 6(8)	11. 5(6)	15. 4(5)	13. 6(5)	8. 0(1)	15. 1(10)	12. 6	2. 2
Precornified	14. 3(11)	15. 0(7)	14. 1(8)	15. 5(10)	16. 6(10)	15. 4(13)	17. 6(10)	14. 0(10)	12. 2(4)	16. 1(10)	15. 1	1. 5
Intermediate	21. 5(2)	18. 8(6)	18. 6(8)	21. 0(1)	22. 4(11)	18. 7(3)	24. 4(8)	21. 0(11)	19. 2(8)	22. 2(6)	20. 8	2. 0
Outer basal		25. 6(5)	22. 6(9)	26. 0(1)	25. 0(2)		33. 3(3)	26. 2(10)	22. 0(7)	25. 0(2)	25. 7	3. 4
Inner basal			33. 0(1)	27. 3(3)	27. 0(1)			29. 0(10)	30. 0(1)	25. 7(3)	28. 7	2. 6

*Parentheses indicate number of cells measured.

From this table it can be seen that there are significant differences between patients. Therefore, the measure of cell types on a comparative basis to other cell types in the same person may be of value in future studies. The estimate of standard deviation for light-emission readings for a given type for a given person is $\sqrt{1.0} = 1.0$. The estimate of standard deviation of light-emission values from one person to another within one cell type is $\sqrt{(8.8 - 1.0)/3} = 1.6$ (*20*). For discriminative purposes by use of the n cells, the standard error will be $\sqrt{(1.6)^2 + (1.0)^2/n} < 1.9$ (for 1 cell). There is some evidence to indicate that cells with higher light-emission values will show somewhat greater variability.



TEXT-FIGURE 3.—Mean light-emission values for different AO fluorochromed cell types in 10 patients with classes AO I and II. Values were extracted from table 6. Solid connecting line represents the mean of the mean emission values for each particular cell type. Critical light-emission value was 36.2 units of galvanometer reading.

TABLE 7.—Analysis of variance of cornified, precornified, and intermediate cells

Source of variation	Sum squares (SS)	Degree of freedom (df)	Mean square (MS)
Patients	79. 0	9	8. 8
Cell types	349. 7	2	174. 9
Residual	18. 0	18	1. 0
Total	446. 7	29	

Table 8 lists the mean emission values for different cell types in AO classes II to V. Degenerated cells were not included in these measurements. However, malignant, naked nuclei were measured because of the significant increase in nuclear DNA and the relative stability in DNA content.

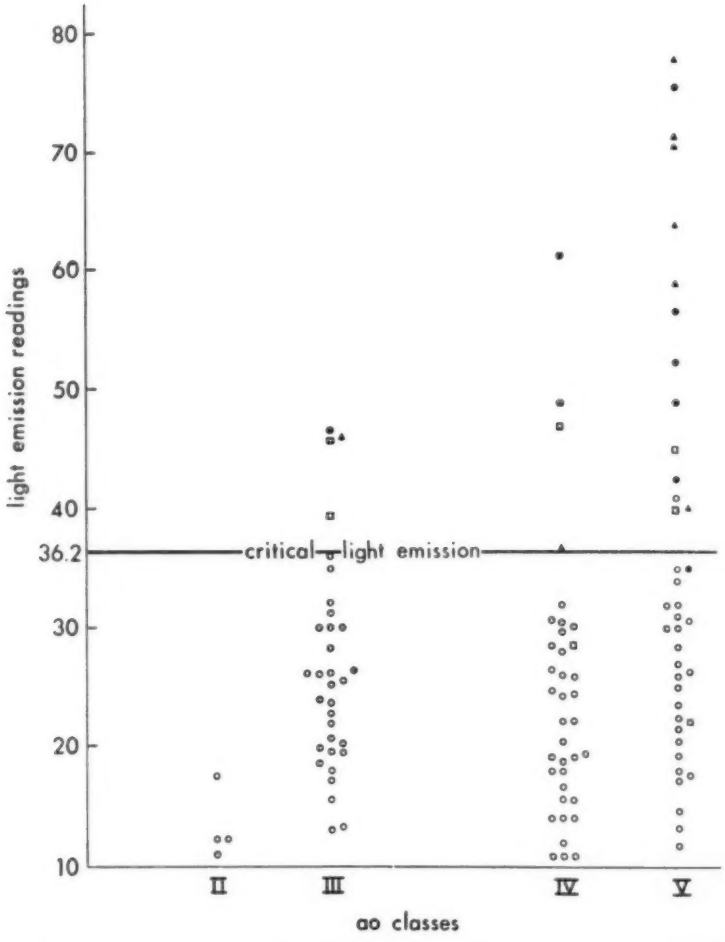
The data of table 8 are represented graphically in text-figure 4. This figure was constructed from the measurement of different cell types in cervical smears of AO classes II to V. We used different symbols for plotting the particular cell types, which were separated on different levels for AO classes II to V. It is evident from text-figure 4 that the critical emission line set at the level of 36.2 units allows for a significant distribution and separation of normal and abnormal cells (text-figs. 3 and 4).

Only 4 readings higher than 36 units for normal cells (inner and outer basal cells) were obtained from smears on 2 patients of AO classes IV and V; 7 elements classified as "suspicious" gave readings below 36 units from smears on patients with AO classes IV and V. The original protocols with measurements and cell description were reviewed, and analysis of the 4 high readings falling into the "normal" category revealed the following: In one instance 3 small degenerating "malignant" cells had been erroneously classified as "normal outer basal cells." This patient had an AO class V smear and a tissue finding with carcinoma *in situ* of the cervix. The one high reading on a "normal inner basal cell" was obtained on one which should have been classified as "atypical" and was from an AO class IV smear, where other abnormal cells gave high emission values. Follow-up smears on this patient 1 year later done elsewhere showed a Papanicolaou class IV. Subsequently (1 month later), cervical tissues by punch biopsies and conization were obtained and exhibited squamous-cell metaplasia with focal areas of atypical hyperplasia and basal-cell hyperplasia, respectively. The pathology report concluded that the degree of cellular atypism in the cervical tissue may well have resulted in exfoliative cytology of class IV, and further observation was recommended. Reviewing from the original protocols the 7 values which fell short of the 36 unit limit in the "abnormal" category, we found that in one instance low readings on 5 atypical cells were obtained from an AO class IV smear from a 74-year-old patient, with senile, menopausal cervical epithelium, where cells exhibited generally low emission values. No tissue confirmation on this patient is available to date. The other low readings from an AO class-V smear were obtained on 2 degenerating cells classified

TABLE 8.—Mean light-emission values for different cell types in AO classes II to V*

AO classes	Cornified cells	Precornified cells	Intermediate cells	Outer basal cells	Inner basal cells	Atypical cells	Suspicious cells	Malignant cells	Malignant, naked nuclei
II		11 (8)	12.4 (5)	17.5 (4)	12.2 (4)				
III	26.2 (4)	25.8 (11)	31.3 (3)	30 (5)	36 (1)	45.8 (7)			
	20.8 (9)	26.2 (11)	28.3 (3)						
	20 (2)	19.7 (3)	26.5 (4)	24 (7)	30 (4)				
				35 (1)					
		13.3 (6)	17.2 (2)	25.3 (6)					
	13 (1)	18 (1)	19.6 (12)	23.7 (7)	32.2 (4)		46.8 (5)		46 (1)
				18.6 (3)	22 (2)				
	15.6 (5)	20.4 (9)	22.9 (9)	30 (1)		39.5 (2)			
IV		18.9 (5)	20.4 (5)	24.6 (5)					36.6 (6)
	19.1 (7)	26.1 (6)		28 (2)		47 (2)			
		14 (2)	19.5 (6)		22.2 (4)	28.6 (5)			
	18 (2)		15.7 (4)	26.6 (8)	24.8 (6)				
	12 (1)	18 (2)	24.3 (7)	29.7 (12)	31.8 (6)				
	11 (1)	16.3 (3)	30.5 (6)	28.5 (13)	49 (1)		61.3 (3)		
	14 (1)	16 (5)	26 (5)	32 (4)	30.1 (6)				
	11 (5)	11 (2)	22.2 (4)		14 (3)				
V		18 (6)	20.5 (2)	30 (1)	32 (1)	45 (1)	56.8 (6)	78 (1)	59 (2)
	27 (1)	30 (2)	34 (1)	41 (3)			75.7 (4)		
	22.5 (2)	23.6 (6)	26.4 (5)	26 (1)	25 (1)	40 (2)	35 (3)		40 (7)
	14.8 (10)	13.2 (10)	22.1 (10)	31.6 (9)	28.4 (7)		52.4 (7)	64.0 (1)	
	17.1 (9)	19.3 (6)	31 (9)	35 (1)	32 (4)		42.6 (6)	71.4 (25)	
	11.9 (8)	17.8 (5)	21.6 (3)				49 (1)	70.7 (4)	

*In parentheses: number of cells measured.



TEXT-FIGURE 4.—Mean light-emission values for different cell types in AO classes II to V. Cell types grouped in categories with these symbols: normal cells (cornified, precornified, intermediate, outer and inner basal cells) ○; atypical cells □; suspicious cells ●; malignant cells (including malignant, naked nuclei) ▲. Mean light-emission values were extracted from table 8.

as "suspicious." These "suspicious" elements, however, might have well been only atypical cells with inflammatory changes such as found in the majority of cells represented in the same smear. Tissue findings (punch) showed "ulcerative chronic inflammation," and we believed that the 2 measured cells probably originated from an inflammatory area adjacent to a malignant focus. In the same smear a single cell and a naked nucleus classified as "suspicious" and "malignant" elements showed higher emission values falling distinctly above the 36 unit level. Follow-up

smears and further tissue studies by conization were indicated to clarify the obtained data.

Analysis of mean emission values on outer basal cells of AO classes II to V indicated a drift toward larger values in higher AO classes (table 9). Calculation of the variance ratio 77.8/22.3 showed this to be significant $F_{.05} = 3.5$ (table 10).

TABLE 9.—Mean emission values for outer basal cells for each patient

	AO class			
	II	III	IV	V
	17. 5	30. 0 24. 0 35. 0 25. 3 23. 7 18. 6 30. 0	24. 6 28. 0 26. 6 29. 7 28. 0 32. 0	30. 0 41. 0 26. 0 31. 6 35. 0
Mean for patient of each class	17. 5	26. 7	28. 2	32. 7

TABLE 10.—Analysis of variance table

Source of variation	Sum squares (SS)	Degree of freedom (df)	Mean square (MS)	F ratio
Between AO classes	233. 28	3	77. 8	$F = 3.49$
Between patients within classes	334. 37	15	22. 3	
Total	567. 65	18		$F_{.05} = 3.29$

Table 11 presents a survey of cytologic classification with cervical tissue findings available at present on 33 patients whose cervical smears were used for single-cell measurements. In the 11 patients with smears of AO classes I and II, tissue confirmation could not be obtained, as this cytologic classification does not justify a request for biopsy in the opinion of the attending physician. Of 8 patients with AO class III smears, tissue studies were made available only on 3. In the opinion of some attending gynecologists who contributed smears on these patients, a class III was not an indication for follow-up by tissue biopsy. Of 8 patients with AO class IV smears, 3 had no tissue confirmation. One patient had a cone biopsy with inconclusive tissue findings; 2 had conization, and the degree of cellular atypia indicated need for further observation; and 2, negative tissue findings by punch biopsy indicated need for conization for further tissue studies. Of 6 patients with AO class V smears, 5 had a conization performed, which revealed carcinoma *in situ* of the cervix. In one patient in whom a punch biopsy was obtained and only ulcerative chronic inflammation encountered, further need for conization was indicated. No invasive carcinoma of the cervix was found in this study.

TABLE 11.—Survey of cytologic classification and tissue findings*†

AO class	Number of patients	Cervical tissue findings
I, II	11	No tissue confirmation
III	8	5 With no tissue confirmation 1 With negative punch biopsy‡ 1 With punch biopsy: chronic cervicitis with squamous-cell metaplasia‡ 1 With punch biopsy: carcinoma *in situ*; however, cells from atypical areas were mostly represented in the examined smear
IV	8	3 With no tissue confirmation 1 With cone biopsy: inconclusive anaplasia versus carcinoma *in situ* 1 With cone biopsy: atypical squamous-cell metaplasia; further observation recommended 1 With punch and cone biopsy: atypical squamous-cell metaplasia, focal basal-cell hyperplasia; further observation recommended 1 With punch biopsy: chronic cervicitis‡ 1 With punch biopsy: atypical squamous-cell metaplasia†
V	6	5 With cone biopsy: carcinoma *in situ* 1 With punch biopsy: ulcerative chronic inflammation‡
Total patients 33		

*Cervical smears on these patients were used for measurement on single cells.

†The authors are grateful to Rodney Carleton, M.D., Pathologist, Kaiser Foundation Hospital, Los Angeles for his helpful cooperation in providing materials and to Leo Kaplan, M.D., Pathologist, Mt. Sinai Hospital and Clinic, Los Angeles, for pathological diagnoses.

‡Further tissue studies by conization were recommended.

DISCUSSION

As established previously (*2*), cancer cells derived from the cervix uteri, in comparison with normal homologous cells, combined with more basic fluorochrome and emitted an average of 2 or 3 times more fluorescence per unit of nuclear area. An increased nuclear chromatin content was present frequently, though not invariably in the preinvasive (*in situ*) carcinoma of the cervix (*21*). The increase in cellular basophilia was considered a valuable criterion in cytologic diagnosis (*22*, *23*), especially when its estimation was combined with quantitative determination of DNA and RNA by a microspectrophotometric device (*23*, *24*). Comprehensive data (*15*) showed the proportional distribution of basophilic and acidophilic cells in smears from dysplasias, carcinoma *in situ*, and invasive carcinoma of the cervix. In view of these reports, a measure of the combined fluorescence emission of the cytoplasmic and nuclear area of the AO fluorochromed cervical cells seemed valuable. The emitted fluorescence of the AO bound by basophilic as well as acidophilic cellular structures covered a broad spectral range from 510 to 700 mμ.

Correlating the microscopic appearance of the cell structure with the identity of its cytochemical components has proved most useful in the understanding of some cellular physiological processes, such as matura-

tion. However, semiquantitative or quantitative techniques performed on different tissues to confirm such correlations are extremely valuable (*25*, *26*). Furthermore, such quantitative information could contribute to a more precise identification of cervical cells exfoliated from "borderline lesions" (*27*, *28*) in which even combined morphologic and cytochemical methods did not establish a clear-cut classification.

A comparison of differences in emission values between cells of the normal and abnormal groups is presented in this study. As mentioned previously, while selecting cells for measurements, we individually described their morphologic and staining characteristics in detail and used them for comparison of their respective emission values with cytologic classification and tissue findings.

The varying number and regional density of available binding sites for AO determined the amount of the bound dye molecules and resulted in varying intensities and hues of cytoplasmic and nuclear fluorescence. Estimated visually, the hues of red (brick, orange) and their different brilliance (dull, bright) indicated relative concentrations of cytoplasmic RNA. In smears of AO classes III, IV, and V, the majority of single, well-isolated measurable elements of abnormal cell types exhibited hyperchromatic nuclei with abnormal morphology, whereas the cytoplasm had a rather low RNA concentration. A relatively smaller proportion of cells had hyperchromatic nuclei and cytoplasm with high RNA concentration. Frequently, such cells of diagnostic importance adhered firmly in clusters and thus were not suitable for measurements. Consequently, more and higher readings were obtained on single abnormal cells exhibiting nuclear hyperchromasia. However, the fact that clusters or small groups of diagnostically important cells could not be included in measurements was a minor disadvantage, in consideration of the laborious procedures required in cell-separation techniques.

Variations in light emission between single normal elements of the same cell type in smears of different classifications (AO class II to V) were partly due to the variable state of cell preservation. The amount of dye retained by cells depended not only on quantitative differences of the available acidic groups of nucleoproteins but also on the integrity of the cell membranes and on the physicochemical alterations after fixation (*13*). Thus, in the applied AO staining procedure a different staining intensity resulted according to the state of cell preservation after the final controlled differentiation step with buffer and calcium chloride had been carried out.

Reviewing the photometric data on normal cells, we found that outer basal cells showed a drift toward larger values in higher AO classes, and analysis of variance proved this to be significant. Enough single elements were not available for a similar analysis on the inner basal cells. However, it seems important to establish in future studies whether younger, less mature elements (inner and outer basal cells) will show in higher classes a consistent drift toward higher emission values, and to analyze the significance of such findings.

A comparison of single-cell measurements with the respective cytologic classification and corresponding tissue findings obtained an apparently good correlation in 5 cases with smears of AO class V, in which tissue sampling by conization was available. However, in another instance, tissue findings (cone) were inconclusive, and in 2 other patients atypical changes found in cone specimens indicated need for further observation. In those patients with smears of AO class III and IV, in which the punch biopsies were negative for malignancy, further tissue sampling by conization was recommended for substantiation or disproval of the cytologic classification (tables 8 and 11).

From emission values falling below or above the dividing line (text-fig. 4) the separation of the normal from the abnormal cell group was clearly evident. With respect to the single higher values of normal cells, or lower readings on abnormal elements, other authors have also encountered overlapping values on exfoliated vaginal cells subjected to cytophotometric estimation (*23*). Customarily, tissue findings are considered the decisive criterion in determination of degrees of epithelial abnormalities in reference to the cellular characteristics in routinely obtained cytologic smears. However, it seems that quantitative information gained by photometric device on single cells can be helpful in situations where neither the cytologic classification nor tissue findings are decisive, and can furnish a more objective basis for estimation of relative differences between varying degrees of cellular abnormalities. It remains to be seen if such quantitative data can be useful also for separation of cells exfoliated from benign, proliferating epithelia, *e.g.*, with reactive changes, inflammation, pregnancy effect, and cells with more severe alterations, such as from atypical squamous metaplasia, severe basal-cell hyperplasia, or carcinoma *in situ*.[7]

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Mammary Carcinogenesis by 3-Methylcholanthrene. III. Induction of Mammary Carcinoma and Milk Secretion in Male Rats Bearing Ovarian Grafts [1, 2]

THOMAS L. DAO *and* MARY J. GREINER, *Roswell Park Memorial Institute, Buffalo, New York*

SUMMARY

These experiments demonstrate that, while mammary cancers do not develop in intact male rats treated with 3-methylcholanthrene (MCA), a few tumors develop when ovaries are grafted into these rats. The tumor incidence rises markedly (14–66%) if ovaries are grafted into castrated male rats. Testicular hormones profoundly inhibit mammary carcinogenesis by 3-MCA. The histologic evidence suggests that ovarian grafts from castrated male rats are subjected to much greater pituitary stimulation than those from the non-orchiectomized males. The ovarian grafts do not survive and function as well in the intact male rats as in the castrated rats. The age of the graft is also important. Transplantation of "young" ovaries in young or old hosts is more successful than the grafting of ovaries from old rats. Grafts of ovaries of old rats are refractory to pituitary stimulation. "Lactation" in the castrated males bearing ovarian grafts is associated with marked enlargement and hyperplasia of acidophils of the pituitary gland. Our experiments support the view that acidophils secrete prolactin. Two factors are involved in the production of pituitary hyperplasia and lactation: (*a*) the effect of gonadectomy, and (*b*) the effect of estrogen produced by the ovarian grafts. The data demonstrate conclusively that in steroid hormone-dependent mammary cancer the neoplasm regresses when the steroid-producing organs, the ovarian grafts, are removed. A hyperfunctioning pituitary, while it is capable of maintaining the secretory activity of the mammary gland, is unable to sustain the growth of a dependent neoplasm in the absence of ovarian steroid hormones. These observations are analogous to those made in humans with hormone-dependent cancers.—J. Nat. Cancer Inst. 27: 333-349, 1961.

PREVIOUS EXPERIMENTS (*1*) demonstrated that 3-methylcholanthrene (MCA) fed to intact male rats failed to induce mammary cancer. Furthermore, in male rats, 3-MCA given orally concentrated in breast tissue essentially the same as in virgin female rats (*2*). Its failure to induce mammary cancer in male rats demonstrates that ovarian hormones are critically important in mammary carcinogenesis by 3-MCA.

Although numerous investigations (*3–6*) have been carried out on induction of mammary carcinoma in mice bearing ovarian grafts, data are

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not available concerning the successful production of mammary carcinoma in male rats by transplantation of ovaries. In the present investigation we studied the effect of ovarian grafts on the incidence of mammary cancer in male rats treated with 3-MCA and the pathophysiological effect of castration and ovarian grafting on pituitary and mammary glands. When extensive secretion of milk in mammary glands occurred in castrated male rats bearing ovarian grafts, these "lactating" animals had acidophil-chromophobe hyperplasia of the pituitary gland, and occasionally the pituitaries showed evidence of early stages of adenoma formation.

MATERIALS AND METHODS

Sprague-Dawley rats were used in all experiments: for controls, 50- to 55-day-old males, and in ovarian transplantation, litter mates of males and females ranging in age from 23 to 120 days.

For ovarian transplantation the ovaries from the female litter mates were removed and inserted subcutaneously into each axilla of the male rats through a small skin incision. In the group in which ovaries were grafted into castrated males, castration was performed at the same time as ovarian transplantation.

The control experiments involved intact males and males castrated at the age of 50 days.

In experiments concerning the effect of ovarian grafts on tumor incidence, ovaries from the female litter mates were transplanted into intact and orchiectomized animals. In the latter group, ovarian transplantation and castration were carried out at the age of 23 to 26, 60, 90, and 120 days.

3-MCA, dissolved in sesame oil at a concentration of 10 mg/ml, was fed daily, 6 days a week for 15 days, to all male rats bearing ovarian transplants either when they were about 50 days old (in animals in which castration and transplantation were done at the age of 23 days) or 2 weeks after transplantation. The carcinogen was given to the castrated animals in the control group for 15 days, 2 weeks after orchiectomy.

The rats were examined at frequent intervals for palpable tumors. We define "latent period of tumor appearance" as the interval from the first feeding with 3-MCA to the detection of the first palpable tumor. The tumors were measured with calipers, and the mean size of each tumor was calculated from the 2 greatest diameters. In one group, when the tumors reached 1 cm in diameter, the ovarian grafts were removed. Subsequently, the growth rate of tumors was observed.

All rats were killed at the end of 6 to 8 months. At necropsy, pituitaries, adrenals, ovarian grafts, seminal vesicles, prostates, and tumors were removed and weighed. Serial histologic sections were prepared for all pituitaries and ovarian grafts. All pituitaries were fixed in Zenker-formol and stained according to the modification of Masson's trichrome technique. The histology of the mammary glands was studied by the wholemount technique.

RESULTS

Incidence of Mammary Cancer in Intact and Orchiectomized Male Rats

The results in table 1 show that all mature female rats developed mammary adenocarcinoma after intragastric instillation of 10 mg/day of 3-MCA for 15 days, but that no males similarly treated developed tumors. When 3-MCA was fed to castrated males, 14 percent of the animals developed mammary cancers.

TABLE 1.—Incidence of mammary cancers in 3-MCA-treated castrated and noncastrated male rats bearing ovarian grafts. Data on comparative tumor incidence are given for intact female and male rats treated with 3-MCA*

Group	Number of rats	Rats with tumors		Appearance of palpable tumors (days)		
		Number	Percent	Range	Median	Mean
♀ Intact	20	20	100	46–135	60	73
♂ Intact	30	0	0	—	—	—
♂ Castrated	27	4	14	70–138	87	98
♂ Intact + ovarian graft†	25	4	16	73–128	122	115
♂ Castrated + ovarian graft†	50	33	66	45–132	88	85

*3-MCA, 10 mg/day, was given all rats for 15 days, 2 weeks after castration and ovarian transplantation.
†Castration and ovarian transplantation were carried out in all animals at the age of 23 to 26 days.

Incidence of Mammary Cancer in Intact and Castrated Male Rats Bearing Ovarian Grafts

When ovaries were transplanted into intact male rats, 4 of the 25 developed mammary cancer. The incidence of mammary cancer, however, greatly increased when ovaries were grafted into castrated males (14–66%) (table 1). This clearly demonstrated that testicular hormones from the nonorchiectomized male rats profoundly affected the development of mammary cancer in the males bearing ovarian grafts. The tumor incidence in the noncastrated male rats with ovarian grafts was similar to that in the castrated males without ovarian grafts. Castrated male rats with ovarian transplants developed mammary cancer earlier than intact males bearing ovarian grafts.

Influence of Age of Ovarian Grafts on Mammary Tumorigenesis

The results are summarized in table 2. From these it is evident that the age of the ovaries grafted into the hosts or the age of the hosts themselves is important in the subsequent induction of tumors in carcinogen-treated male rats. The tumor incidence was 66 percent when young ovaries were grafted into young hosts 25 to 30 days old, but tumors failed to develop when ovaries were transplanted into rats at the age of 120 days. Since the animals used were litter mates, we did not perform

any experiments in which young ovaries were transplanted into aged hosts or vice versa. However, when ovaries from 5 nonlittermate females, 35 days old, were transplanted into five 6-month-old castrated male rats, the ovaries appeared normal histologically. This indicates that transplantation of "young" ovaries into aged hosts is more successful than transplantation of ovaries from aged donors to old hosts.

TABLE 2.—Influence of age of ovarian graft on incidence of mammary carcinogenesis induced by 3-MCA*

Group	Number of rats	Age at castration and grafting (days)	Number of rats with tumors	
			Number	Percent
1	50	23–26	33	66
2	12	60	3	25
3	13	90	1	7
4	8	120	0	0

*3-MCA, 10 mg/day, was given all rats for 15 days, 2 weeks after castration and ovarian transplantation.

Induction of Milk Secretion in Mammary Glands of Castrated Male Rats Bearing Ovarian Grafts

In a total of 50 castrated male rats bearing ovarian transplants, 30 (60%) developed moderate to extensive milk-cyst formations in inguinal and axillary breasts (fig. 1). Of these 30 with milk cysts, 20 also developed mammary cancers.

We observed that in these "lactating" castrated male rats the pituitary glands were uniformly and significantly enlarged (table 3). Histologically, the acidophilic and chromophobe cells were markedly increased in number and size. Large sinuses filled with red corpuscles were frequently seen (fig. 2). In 5 animals, the histology of the hyperplastic pituitary resembled adenoma. Cords of hyperplastic acidophilic and chromophobe cells were centrally located in the anterior lobe and formed a definite nodule; the cells in the periphery were small and crowded into a narrow zone. No "castration cells" were seen in any of these pituitaries.

Although the adrenal glands of these "lactating" male rats were enlarged, definite hyperplasia of the cortex was not observed histologically. However, there were numerous dilated blood vessels in the zona reticularis.

Effect of Removal of Ovarian Grafts on Growth Rate of Mammary Cancers

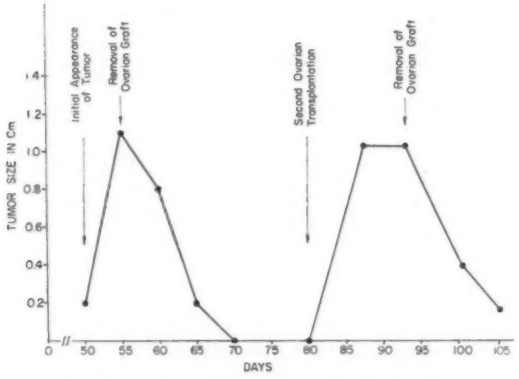
Ovarian grafts were removed from 10 castrated male rats when their mammary cancers reached more than 1 cm in diameter. The mammary tumors regressed and became nonpalpable in all these animals after removal of the ovarian grafts. When fresh ovaries from 5 nonlittermate female rats, 35 days old, were transplanted into 5 castrated males with regressing mammary tumors, after the removal of the original ovarian

TABLE 3.—Pituitary and adrenal weights in male rats bearing ovarian transplants*

Group	Number of animals	Pituitary weight (mg)			Combined adrenal weight (mg)		
		Range	Median	Mean†	Range	Median	Mean†
Intact‡	20	11.4–18.4	14.0	14.2 ± 2.0	45.4–75.6	62.4	62.2 ± 7.7
Intact + ovarian transplant	16	9.0–18.0	13.4	13.8 ± 2.7	44.0–81.0	62.0	63.3 ± 12.2
Castrated	23	12.2–27.4	21.0	20.8 ± 3.7	46.8–74.0	59.4	60.2 ± 6.9
Castrated + ovarian transplant	18	10.0–26.4	17.0	17.6 ± 4.0	52.4–79.0	65.6	66.7 ± 8.2
Castrated + ovarian transplant + tumor	18	14.0–27.0	17.6	18.8 ± 3.4	60.4–92.0	70.6	72.6 ± 9.3
Castrated + ovarian transplant → "lactation"	10	18.0–36.8	25.6	25.9 ± 5.7	70.0–110.0	82.6	85.1 ± 12.0
Castrated + ovarian transplant → "lactation" + tumor	20	18.0–56.6	23.6	25.5 ± 8.6	62.8–101.6	85.4	85.3 ± 9.6

*3-MCA, 10 mg/day, was given to all animals for 15 days.
†± Standard deviation.
‡Nonorchiectomized rats without ovarian transplant.

grafts, we observed resumption of tumor growth in all 5 rats 1 week after the second graft was implanted. These tumors were allowed to grow until they were again larger than 1 cm, and the second pair of ovarian grafts was removed. These tumors again regressed (text-fig. 1) to non-palpable size and did not reappear at autopsy, a month after the removal of the second pair of grafts. In the other 5 animals in which a second pair of ovarian grafts was not transplanted, all tumors remained non-palpable 2 months after removal of the original grafts, when autopsies were performed. These experiments clearly demonstrated that ovarian grafts were a *sine qua non* to the maintenance of mammary-tumor growth.



TEXT-FIGURE 1.—Mammary tumor developed in castrated male rat bearing ovarian graft and regressed after removal of transplant. *Note* resumption of growth of tumor after a new pair of ovaries was implanted and regression again after removal of graft.

Histology of Ovarian Grafts

All ovarian grafts removed at autopsy were sectioned serially and studied. Ovarian grafts were recovered from 19 of 25 intact male rats bearing transplants. Among these 19 grafts, only 5 pairs appeared normal. Enormous cystic enlargement of the transplants was characteristic in the nonfunctioning ovarian grafts in the remaining intact males. Macroscopically, these grafts often contained only one or two large cysts, with little normal ovarian tissue. Microscopically, the cysts were large and lined with a single layer of flattened epithelial cells. Ovarian follicles and corpora lutea were never observed. Ovarian stromata in these grafts were scanty (fig. 3). The lack of estrogen effect was further evidenced by normal testes and prostates.

Ovarian grafts were recovered from 48 of the 50 castrated male rats autopsied. Grossly the grafts were often cystic, usually with multiple small follicular cysts. Histologically, the surviving grafts contained primordial and large mature follicles, and in some instances corpora lutea. The stromata in these functioning ovarian grafts were abundant (fig. 4). The survival and function of the grafts were *pari passu* with the failure of

"castration cells" to appear in the pituitary (fig. 5). The seminal vesicles in castrates bearing functional ovarian grafts showed a significant increase of weight (range 78.2–112.0 mg, mean 93.7 mg) above those from the castrated males without ovarian grafts (15.2–26.4 mg, mean 23.6 mg). The weights of seminal vesicles from castrated rats bearing a nonfunctioning ovarian graft were similar to those of the castrates without ovarian transplants. Histologically, there was definite connective-tissue metaplasia in the seminal vesicles from castrated rats bearing functional grafts. It has been suggested that estrogen stimulates connective-tissue metaplasia (7).

The ovarian grafts recovered from castrated male rats developing "lactation" showed one important difference, *i.e.*, the frequent presence of corpora lutea, which had increased, and were easily recognizable even on gross examination. This increase was certainly due to the augmented luteotrophic hormone production by the hyperplastic pituitary in the "lactating" rats. No granulosa-cell tumor was ever observed in the ovarian grafts.

The 5 pairs of ovarian grafts removed from nonlitter mates all had the appearance of normal ovaries.

Although castrated male rats bearing functional ovarian grafts did not all develop mammary cancers, the results indicated that a functional ovarian transplant was essential to the induction of mammary cancer after the administration of 3-MCA.

Morphology of Mammary Glands in Male Rats Bearing Ovarian Transplants

Mammary glands of rats from different experiments were studied by the wholemount technique.

The mammary glands of 65-day-old noncastrated males showed ductal and alveoli development. The mammary-gland development in intact male rats was very similar to that of females at a comparable age.

After orchiectomy, the mammary glands involuted gradually; the ducts became slender, and there was a significant decrease in budding 2 months after castration (fig. 6). We did not observe such changes, however, in orchiectomized male rats bearing ovarian transplants. In these animals, the mammary glands were essentially similar to those of noncastrated males and mature females. In castrated male rats having "lactation," the mammary glands were extensively developed, which was apparent even macroscopically. The ducts were filled with secretion and the alveolar luminar were distended (fig. 7).

DISCUSSION

One of the most significant findings is the great difference in the development of mammary cancers in the two groups of male animals, noncastrated and orchiectomized, both bearing ovarian grafts. The low

incidence of mammary cancer in the intact males bearing ovarian transplants provides conclusive evidence that normal testes profoundly inhibit the induction of mammary cancer by 3-MCA. Our experiments indicate that the presence of normal testes interferes with the survival and function of the ovarian grafts. Although the exact mechanism of the inhibitory effect of the testicular hormones on function of the ovarian grafts is not clear, the histologic evidence seems to suggest that grafted ovaries in castrated male rats are subjected to greater pituitary stimulation than those in noncastrates. This lack of pituitary stimulation is attributable to the inhibition of pituitary gonadotrophic activity by testicular hormones.

The survival and function of the ovarian grafts seem to depend also on the age of the ovaries. The results of our experiments indicate that the age of the grafts is one of the determining factors. The transplantation of "young" ovaries to young or old hosts is more successful than the grafting of ovaries from old rats. Although we have not carried out extensive experiments to study the survival of "young" grafts in old hosts, 5 pairs of ovaries from nonlittermate young rats, when transplanted to 5 hosts 6 months old, survived and functioned normally. It is suggested that the "old" ovaries probably are more refractory to stimulation by the hypophysis. Burack and Wolfe (*8*) demonstrated that the ovaries of old rats were less responsive to stimulation by homologous anterior pituitary implants than were the ovaries of young rats. Wolfe (*9*) also experimented with exchanging ovaries between pairs of rats, one young and one old, by reciprocal transplantation into the kidneys. He found, on the basis of histologic examination, that "young" ovaries survived in old animals better than "old" ovaries in young rats.

Huseby and Bittner (*5*) earlier reported similar observations regarding the incidence of spontaneous mammary cancer in male mice bearing ovarian grafts. These authors noted that the ovarian transplants took in both noncastrated and castrated male mice, but mammary-gland development was markedly different in these two groups. In the castrated males with ovarian grafts, the mammary glands underwent extensive ductal and alveolar development, in contrast to the totally undeveloped mammary glands in noncastrated mice with functioning ovarian grafts. In the present experiments, the mammary glands of male rats, in contradistinction to mice, show a development parallel with that of the female glands. There are both duct-system extension and alveolar development. The decrease in incidence of mammary cancer in noncastrated male rats therefore cannot be ascribed to the lack of a developed mammary gland.

Induction of milk secretion in the mammary glands of ovariectomized mice and rats bearing ovarian grafts has been previously reported (*5*, *10–12*) but no similar observation has been made in male castrates bearing ovarian transplants. In the present experiments, the induction of "lactation" in the mammary glands is certainly due to the increased prolactin secretion as a result of acidophil-chromophobe cell hyperplasia.

From histologic examination of these pituitaries, it becomes evident that the hyperplastic cells have aggregated into a mass resembling the early stages of an adenoma which will eventually form if the rats are allowed to live longer. Bielschowsky (*10*) reported functional acidophilic pituitary tumors causing lactation in an ovariectomized rat, an intact female joined in parabiosis to a castrated partner, and a female treated with diethylstilbestrol. The estrogen-induced mammotropic tumors described by Furth (*13*) are characteristically acidophilic. Whatever the mechanism of the induction of lactation may be, one common denominator, the acidophilic hyperplasia or tumor, is always present. The results of our experiments support the view that acidophils secrete prolactin. Clearly, two factors are involved in the production of pituitary hyperplasia and lactation: (*a*) Gonadectomy contributes to the continuous stimulation of gonadotrophs by gonadal hormone deficiency and, finally, pituitary hyperplasia or adenoma formation (*12*), and (*b*) estrogen is produced by ovarian grafts under pituitary stimulation.

It is known that the estrogenic substances and corticosteroids are essential to the development of milk-secreting tissue, as is also a protein (*14*), the lactogenic hormone that induces milk formation in the mature breast. Whether estrogen acts indirectly on the mammotrope of the pituitary to secrete "mammotropin," which stimulates the mammary gland to proliferate and to lactate as postulated by Furth, is a matter of conjecture since conclusive evidence is still lacking. Furthermore, the observation of induction of milk secretion in breasts of gonadectomized female rats (*10*) and in gonadectomized and adrenalectomized male and female rats (*12*) having acidophil-chromophobe adenomas indicates that estrogen may not be essential for the stimulation of acidophils to secrete prolactin. In these experiments, it seems that the production of the acidophil-chromophobe adenoma is ascribed to the deficiency of gonadal hormones. That the mammary glands in these animals develop and secrete in the estrogen-deficient environment does not necessarily mean that pituitary hormones can induce mammary-gland development without the participation of ovarian hormones. It is more likely that the potential secretory capacity can be retained by the matured breasts of these animals in a steroid-deficient environment. In Bielschowsky's experiment, the rat was pregnant 4 times and then castrated at the age of 15 months. It is doubtful that a complete regression of the previously overstimulated gland will ever occur after castration. Huggins and Dao (*15*) demonstrated that many women with cancer of the breast, even those in the seventh decade, secreted milk when the mammary gland was challenged with luteotrophin despite the menopause and earlier removal of the ovaries. It is evident that a potential secretory capacity can be retained by the breasts of the human female in senility.

Our experimental results also show an interesting parallelism to a clinical observation, reported earlier by these same authors (*15*), of the induction of lactation by luteotrophin in a man who was castrated and given estrogen for advanced cancer of the prostate. This man continued to lactate for

7 years without maintenance with luteotrophin. Dao (*16*) later reported that "lactation" induced in women with mammary cancer persisted after both ovaries and adrenals were removed. Hypophysectomy successfully caused cessation of "lactation" in these patients, but no regression of mammary cancer was ever observed.

In previous papers (*1*, *2*), we discussed the role of hormones in mammary carcinogenesis induced by 3-MCA. In a dependent mammary cancer, ovarian hormones are not only important in promoting the appearance of the tumor, but are necessary for the maintenance of its continued growth. Our present experiments again conclusively demonstrate that it was the hormones from the grafted ovaries that were sustaining the tumor growth, since the removal of those ovaries caused total regression of the cancer. The prolactin secreted by the hyperfunctioning pituitary gland, however, is capable of maintaining the secretory activity of the mammary gland but is unable to sustain the growth of a neoplasm which is dependent on the presence of ovarian steroids.

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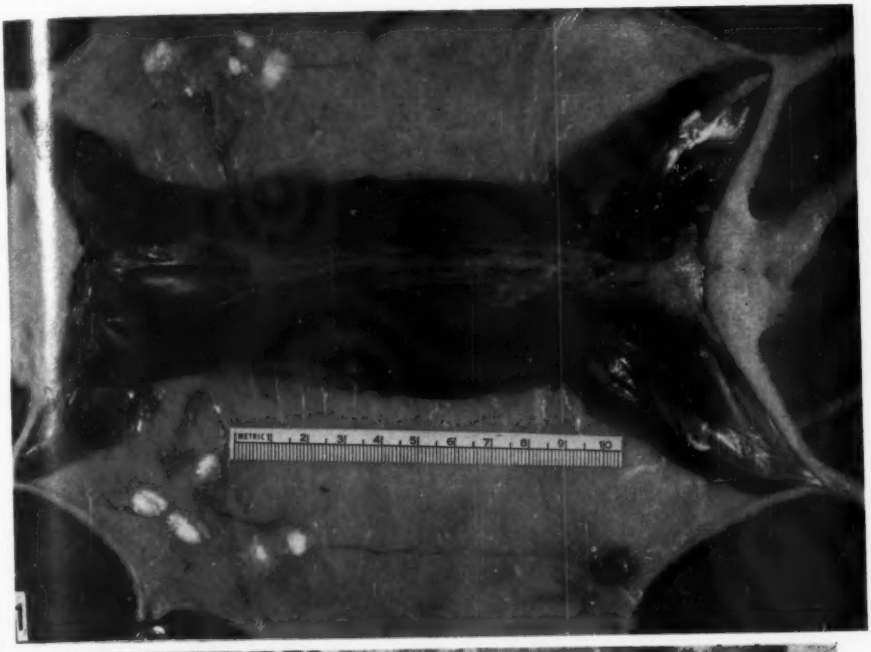
PLATE 39

FIGURE 1.—Moderate number of milk cysts forming in breasts of castrated male rat bearing ovarian grafts. *Note* also development of mammary adenocarcinoma in right inguinal breast.

FIGURE 2.—High-power view of area of hyperplastic pituitary gland of "lactating" male castrate bearing functioning ovarian grafts. *Note* numerous large acidophils with granules and characteristic absence of "castration cells." × 1,500



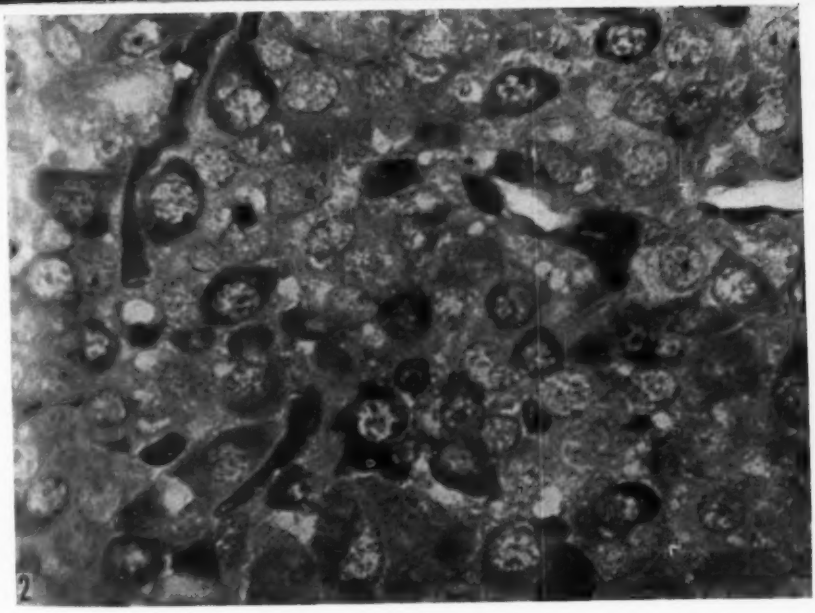


PLATE 40

FIGURE 3.—Nonfunctioning ovarian graft removed from intact male rat. *Note* 3 large cysts lined with flattened epithelial cells. Ovarian stroma are scanty and follicles and corpora lutea are not present. × 45

FIGURE 4.—Functioning ovarian graft removed from castrated "lactating" male rat. *Note* numerous primordial and mature follicles and corpora lutea. × 45

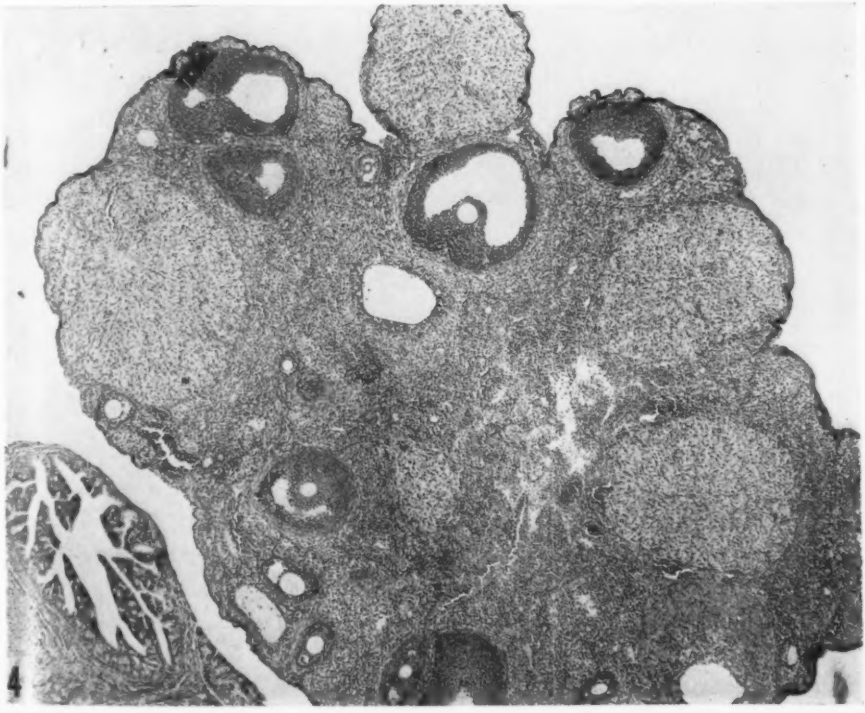
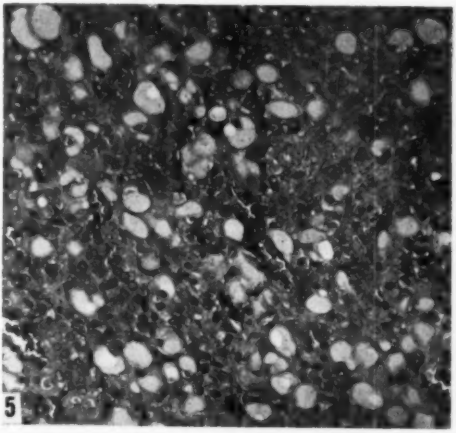


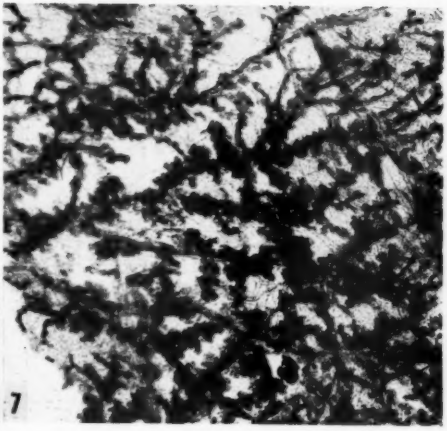
PLATE 41

FIGURE 5.—Area of pituitary gland of castrated male rat bearing nonfunctioning ovarian grafts. *Note* characteristic presence of numerous "signet ring" cells.

FIGURE 6.—Photomicrograph of section of wholemount of mammary gland of male rat 4 months after castration. *Note* slender ducts and total lack of alveolar development. × 50

FIGURE 7.—Photomicrograph of section of wholemount of mammary gland of castrated male rat bearing functioning ovarian grafts. *Note* extensive duct-alveolar development. Alveoli are distended with secretion. × 50





Comparisons Between Survival of Grafted Skin, Ovaries, and Tumors in Mice Across Histocompatibility Barriers of Different Strength [1, 2]

OWE E. A. LINDER,[3] *Institute for Tumor Biology, Karolinska Institutet Medical School, Stockholm, Sweden*

SUMMARY

The survival of grafted skin, ovaries, and tumors in mice was compared in various donor-recipient combinations involving weak (*non-H-2*) histocompatibility differences. Skin grafts were uniformly rejected while tumor grafts readily transgressed the weak barriers which could nevertheless be revealed by small inoculums and preimmunized recipients. Ovarian grafts showed a behavior intermediate in degree between skin and tumor grafts. They often, but not always, survived in spite of the weak histoincompatibility and, although infiltrated with inflammatory cells, continued to function during the entire observation period of 250 to 400 days. Although skin was uniformly rejected in untreated animals of the same donor-host systems, it showed an unexpectedly high rate of survival when grafted upon animals carrying a surviving ovary from the same donor genotype. Random selection of genetically compatible recipients could be safely excluded, and the findings were viewed as possibly related to such phenomena as acquired tolerance, immunological enhancement, and immunoparalysis.—J. Nat. Cancer Inst. 27: 351-373, 1961.

THE NUMBER of histocompatibility factors that differentiate 2 inbred strains of mice is usually estimated by tissues grafted from the 2 strains to their F_2 or backcross hybrid progeny (*1–3*). In a previous work the F_2 test was applied to the "coisogenic resistant" (IR) strains A/Sn and A.SW/Sn (*4*). The IR lines have been produced by Snell (*5–7*) and, under ideal conditions, they tend to become "homozygous lines genetically identical or isogenic except for one histocompatibility locus, at which locus the allele present is different in the two lines" (*6*).

Even if preimmunized hybrids were used for tests with various kinds of tumor grafts, the results were in good agreement with the expected one-gene difference. Skin grafts, on the other hand, which are more sensitive indicators of weak histocompatibility differences than tumor grafts, revealed that the two strains differed at not less and probably more than 10

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loci. In addition, they gave evidence of residual heterozygosis within the A.SW line that regularly rejected intrastrain skin grafts while such grafts were uniformly successful within the A strain itself and other standard inbred lines (*8*).[4] In contrast, carcinomas, sarcomas, and lymphomas of A.SW origin grew progressively in nearly 100 percent of isologous recipients, even if the hosts were preimmunized 10 days earlier by a single intraperitoneal injection of a tissue suspension derived from a mixture of kidney, liver, and spleen of the same genotype as the grafted tumor.

This paper contains further transplantation studies with these strains and 2 additional IR lines that also have a strain A background (A.CA and A.BY). The purpose of the present work was: 1) to estimate the number of histocompatibility-gene differences separating the IR sublines A.CA and A.BY from the A strain by grafts of parental skin to F_2 hybrids; 2) to study the sensitivity of ovarian grafts toward the weak (*non-H-2*) histocompatibility differences, which separate A and A.SW, and to compare them to skin and tumor grafts; 3) to make further efforts to reveal the weak barriers by tumor grafts under more stringent experimental conditions, *i.e.*, by the inoculation of low tumor-cell numbers into preimmunized recipients.

Ovarian grafts were chosen for comparison with skin and tumor transplants because of: 1) the easy demonstrability of surviving grafts by virtue of their ability to maintain the estrous cycle in ovariectomized recipients and 2) the possibility that ovarian grafts were less vulnerable by weak homograft reactions in comparison with skin, as indicated by the studies of Krohn and Hicken and Krohn (*9*, *10*).

MATERIALS AND METHODS

Mice.—Four IR lines of mice were used. They have been developed by Dr. G. D. Snell (*5*, *6*) who kindly sent us breeding pairs in March, 1955. All 4 lines have a strain A background but differ with respect to the *H-2* locus. The original A/Sn line (genotype $H\text{-}2^aH\text{-}2^a$) was derived from the Strong A strain, established in 1921 (*7*). According to the "Standardized Nomenclature for Inbred Strains of Mice" (*11*), it had undergone 87 generations of inbreeding up to 1952. The A/Sn subline was in its 19th generation when we received it and is currently in the 31st brother × sister generation in our laboratory. When received, the IR subline A.SW ($H\text{-}2^sH\text{-}2^s$) was in the 24th, A.BY ($H\text{-}2^bH\text{-}2^b$) in the 17th, and A.CA ($H\text{-}2^fH\text{-}2^f$) in the 18th generation. They were bred by continuous single-line brother × sister mating from their 12th generation. In our laboratory the strains are maintained by brother × sister mating, regularly interrupted by interpolated backcrossing to the A strain after

[4] After the present work had been terminated, the origin of this heterozygosis was traced by Dr. Snell to a contamination of the A line used for his backcrosses during the early history of the IR lines. This will be discussed later.

each 3 or 5 brother × sister generation. On these occasions mice of the sublines A.SW, A.CA, and A.BY, respectively, are crossed with A/Sn animals. F_2 mice derived from these crosses are subsequently challenged with tumors of A/Sn origin, and the survivors are antigenically typed by the hemagglutination test of Gorer and Mikulska (*12*). Mice showing the *H-2* isoantigenic type of the particular IR subline entering the cross are subsequently used to replace the breeding pairs of this line, and their progeny are bred by brother × sister crossing for another 3 to 5 generations. This repeated backcrossing to the A/Sn strain was introduced to maintain as nearly as possible the identity of the common genetic background in all 4 lines. At the time of these experiments, a total of 11 or 12 brother × sister crosses had been made since the mice were received from Dr. Snell. Backcrossing to the A/Sn strain preceded the first and the fifth brother × sister cross.

In addition to mice of the 4 IR lines, various F_1 and F_2 hybrid combinations were also used, including outcrosses of A and A.SW with C57BL and C57L. All grafted recipients ranged from 18 to 25 g in weight and were 3 to 4 months of age. The mice were kept on a standard diet in pellet form; drinking water and pellets were available *ad libitum*.

Tumors.—Three tumors of A.SW and 1 of strain-A origin were used. The A.SW tumors included a spontaneous lymphoma (WL4), a spontaneous mammary carcinoma from a breeding female (S3W), and a methylcholanthrene-induced sarcoma (MWE). The A tumor was a spontaneous lymphoma designated SL1. All tumors were carried in isologous recipients for 13, 1, 15, and 10 generations, respectively. During intermittent periods of varying length the tumors were preserved in our frozen tumor bank. In previous studies (*13*) frozen preservation did not affect tumor histocompatibility in any detectable way.

H-2 *typing by hemagglutination.*—The *H-2* type of segregating hybrids was determined by the hemagglutination method of Gorer and Mikulska (*12*). The mice were bled from the retro-orbital sinus, and washed erythrocyte suspensions were tested against known isoantiserums. Each mouse was typed once—on two different occasions.

Skin grafts.—The method of operating and the criteria used for determining the survival time of a skin graft have been described previously (*4, 14*). A few details can be added here. The visual diagnosis was confirmed in about 60 percent of the animals by histological examination of biopsied material. Particular search was made for signs of the lymphocytic infiltration that usually precedes or accompanies the homograft reaction. It was also expected that if small subpannicular remnants were present they could be detected histologically. An obvious shortcoming of this method is that serial observations are not generally possible on the same animal. For this reason a simple modification of the ordinary inspection method suggested by Taylor and Lehrfeld (*15*) was used in about 60 percent of the mice. A drop of mineral oil was placed between the skin graft and an object glass. A magnification of 10 to 25 times could be obtained under a binocular dissecting microscope. Incipient homo-

graft reactions, not yet apparent to the naked eye, were often indicated by distended blood vessels and local hemorrhages. A new type of bandage gave satisfactory results. Instead of vaseline-impregnated gauze compresses and a plaster of Paris bandage as suggested by Billingham and Medawar (*14*), an acryl plastic bandage, Nobecutan (Nobelkrut, Bofors, Sweden), was sprayed on and around the graft in 3 or 4 thin layers. Since it is absolutely necessary to spray on a dry surface, the grafted area was first dried with an electric lamp. The plastic bandage is bactericidal and fungicidal and permits the skin to perspire. It is quite transparent and the grafts can be regularly inspected. Sometimes the mice scratched the surface of the plastic film, but this did not affect the survival of the graft. The plastic bandage was especially suitable for small grafts of the type used (0.8–1.0 cm^2).

Ovarian grafts.—Adult females 3 to 4 months old were used both as donors and recipients. The recipients were anesthetized by an intraperitoneal injection, 0.06 to 0.07 mg/g, of Nembutal (Abbott Laboratories, Chicago, Ill.), and both ovaries, together with the distal part of the uterine horns, were removed through dorsolumbar incisions. The excised tissue was carefully examined under a dissecting microscope to check the complete removal of the ovaries. In the earlier experiments the ovarian vessels were not ligated, but ligation, introduced later as a regular procedure, avoided heavy bleeding and decreased mortality from about 5 percent to 0.

Each castrated mouse received one ovarium cut in two pieces. As suggested by Krohn (*9*), the ovaries were placed on a moistened filter paper under the dissecting miscroscope and cleaned of connective tissue and remnants of the tuba. Each ovarian half was introduced into a groin near the big femoral vessels. The cut surface of the halved ovaries probably provides a better surface area for revascularization than whole ovarian grafts which are affected more by the early ischemic state described by Harris and Eakin (*16*), but the number of oocytes must be reduced after such an operation. According to Stevens (*17*), however, bisected ovaries are more successfully grafted than either whole or quarter ovaries. The transplanted ovarian halves were usually in their new position 3 to 4 minutes after the donor animal had been killed.

According to the suggestion of Ferguson and Kirschbaum (*18*), the recipients were given 12 IU chorionic gonadotrophin intramuscularly (Pregnyl, 120 IU per ml, Pharmacia, Uppsala, Sweden) daily during the first 2 days after transplantation.

The fate of the ovarian graft was determined by (*a*) a functional test and (*b*) histology. The functional test was based on an assessment of estrogenic activity in the host. The estrous cycle was studied by the vaginal-smear method (*19*, *20*); the whole cycle was carefully followed through all stages. This was preferable to the recording of the days of estrus, since estrus may last for a few hours only and can be easily missed unless expected. Vaginal smears were taken daily, beginning on the 9th day after operation, with the broad end of a flattened tooth pick. No

detectable irritation of the vaginal epithelial cells was observed. The smears were stained by the Papanicolaou method (*21*). The day when no cornified or nucleated epithelial cells were found and there was an abundance of leukocytes was taken as the last day of estrogen production. For histological examination, ovaries or their remnants were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The sections were examined particularly with regard to primordial, graafian, atretic, and cystic follicles and the appearance of corpora lutea. Degenerative processes were accompanied by lymphocytic infiltration, impaired vascularization, and necrosis. When the ovarian transplant could not be found at autopsy the graft was recorded as rejected.

Grafting of neoplastic tissues.—The tumors were brought into suspension by being forced through a 60-mesh stainless-steel screen into 2 volumes of Ringer's solution containing 100 IU penicillin and 100 μg streptomycin/ml. This was followed by filtration through sterile gauze. The proportion of eosin-unstained cells was determined (*22*) and, after appropriate dilution, aliquots containing known cell numbers were inoculated subcutaneously into the right flank. Developing tumors were inspected daily and measured by caliper. Mice dying with progressively growing tumors were registered as positive; those surviving for more than 3 months without tumor development as negative.

RESULTS

Transplantation of Skin

Interstrain, intrastrain, F_1, and F_2 hybrid grafts.—Skin grafts were made within the IR lines and from the A and A.SW lines to their F_1 and F_2 hybrids. The results are summarized in table 1 which also includes the summary of the earlier data, obtained during January to August, 1959. In accordance with the earlier results, the A strain was fully tolerant of intrastrain skin grafts while the 3 IR lines showed evidence of residual heterozygosis, expressed by the rejection of intrastrain grafts. The mean survival time of rejected skin grafts was different for the various combinations, in good agreement with the data obtained about 1 year ago. Skin grafting was also carried out on combinations not tested before. Thus 29 (A.SW $\times$ A.BY)F_1 hybrid mice were grafted with skin from isologous donors. One recipient died 120 days after skin transplantation from a subsequently inoculated tumor and was then still carrying a healthy skin graft. All others rejected their grafts within 12 to 65 days, with a mean survival time of 26.2 $\pm$ 1.9 days.

A similar experiment was carried out with 10 other (A.SW $\times$ A.BY)F_1 hybrids which rejected the grafted F_1 skin after 25.3 $\pm$ 2.7 days. After an interval of 60 days they were grafted with (A.SW $\times$ A.BY)F_1 skin for a second time. This time the skin transplants were sloughed earlier, the mean survival time being 16.1 $\pm$ 1.5 days.

TABLE 1.—Survival in days of inter- and intrastrain skin grafts

Donor	Recipient	Grafting performed during January to August, 1959 (4)			Grafting performed during August, 1959, to June, 1960		
		Mean survival time ± SE	Permanent survival	Number of mice	Mean survival time ± SE	Permanent survival	Number of mice
A	A		10	11*		15	15
A.SW	A.SW	25.2 ± 6.0	—	5	28.0 ± 4.7	—	20
A.BY	A.BY	43.2 ± 4.7	—	6	44.6 ± 5.6	—	15
A.CA	A.CA	46.8 ± 14.8	—	6	45.7 ± 8.8	—	10
A.BY	(A × C57BL)F_1	43.0 ± 3.2	—	8	43.5 ± 2.8	—	20
A.SW	(A × A.SW)F_1	25.7 ± 5.7	—	7	24.9 ± 5.3	—	15
A	(A × A.SW)F_2	24.6 ± 1.6	—	74	23.0 ± 2.5	—	19
A.SW	(A × A.SW)F_2	22.2 ± 1.7	—	83	21.9 ± 2.0	—	20

*One mouse rejected its skin graft after 45 days. Unfortunately it died when the skin was being grafted the second time.

Skin grafts were exchanged between 10 strain A and 10 A.SW mice. The mean survival time of strain A skin on A.SW was 11.1 ± 0.3 days while A.SW skin survived for 11.3 ± 0.5 days on A.

Survival of A, A.SW, A.BY, and A.CA skin on F_2 hybrids.—The results are summarized in table 2. In agreement with the previous findings (A × A.SW)F_2 hybrids invariably rejected A or A.SW skin grafts. The same was true for A.SW skin grafted to (A.SW × A.BY) or (A.SW × A.CA)F_2 hybrids in the present experiments. In all other strain combinations, however, a small fraction of the skin grafts survived permanently (more than 150 days) on the F_2 recipients. The approximate number of histocompatibility factors separating the two strains entering the cross can be estimated from this fraction. With A and A.BY skin grafted to (A × A.BY)F_2 hybrids, permanent survival was obtained in 9.4 and 6.0 percent, respectively. This suggests that 8 to 10 histocompatibility genes are segregating. Survival of parental skin grafts on (A × A.CA)F_2 and (A.CA × A.BY)F_2 hybrids, respectively, indicated that the number of histocompatibility gene differences between A and A.CA were about 11 to 12 and those between A.CA and A.BY about 14 to 15. These figures must be taken with caution, however, since all parental strains except A were characterized by residual heterozygosis, which means that even the donors from these strains were segregating with regard to an unknown number of histocompatibility genes. The estimates are therefore only tentative and indicate the possible order of magnitude of the number of gene differences involved.

Transplantation of Ovarium

Estrogenic activity as a functional test.—The normal estrous cycle was followed by daily vaginal smears in 15 (A × A.SW)F_2 mice 3 months old at the beginning of the experiment. As a rule the mice came into estrus

every 3d or 4th day, and only on 8 occasions during an observation period of 3 months were the cycles extended to 5 days in different recipients.

After 20 (A × A.SW)F_2 females were spayed, all animals entered permanent diestrus 1 day after operation.

Ten (A × A.SW)F_2 hybrid mice were spayed and immediately thereafter autografted with 2 ovarian halves. After a latent period of 9 to 12 days all transplants functioned well as far as could be judged from the re-establishment of the estrous cycle. The frequency of estrus was somewhat irregular in the beginning and the interval between the first and the second scored estrus was in 40 percent of the mice extended to 6 to 7 days. In less than 3 weeks, however, all autografted mice behaved in exactly the same way as the untreated females. The observation period comprised 120 days.

Ovarian autografts of 10 (A × A.SW)F_2 hybrid mice, found to maintain regular estrous cycles during an observation period of 70 days, were excised. No new estrus developed subsequently and there was a reversal of the vaginal smears to those found in the castrated mice.

Isografts and homografts.—Ten castrated (A × C57L)F_1 females were grafted with strain A ovaries. After a latent period of 9 to 10 days all recipients came into estrus. They showed regular estrous cycles of about 4 days during the whole observation period (*cf.* table 3, expt. 1).

Subsequent to ovariectomy, 8 strain A and 8 A.SW mice were homografted with bisected ovaries from A.SW and A donors, respectively. On the following day all recipients showed diestrus and no estrus was seen subsequently in any mouse.

To test the ability of an ovarian transplant to survive and function in spite of a weak (*non-H-2*) histoincompatibility, strain A.BY ovaries were grafted to ovariectomized (A × C57BL)F_1 hybrids. In contrast to skin grafts, which were all ultimately rejected, the transplanted ovaries functioned well for more than 150 days in all 20 recipients tested (table 3). Analogous results were obtained for the *non-H-2* differences that separate some of the IR lines used. When (A.SW × A.BY)F_1 tissue was transplanted to (A.SW × A.BY)F_1 recipients or A.SW tissue to (A.SW × A.CA)F_1 recipients, all skin grafts were regularly rejected while all ovarian grafts tested (6 and 3, respectively) were permanently accepted.

Another comparison between skin and ovarian grafts was performed with (A × A.SW)F_2 mice grafted with tissues from both parental strains. The skin-graft data were taken from the previous work (*4*), which seemed justifiable because of the good reproducibility of the data (table 1). The *H-2* type of all recipients was determined serologically. The results are shown in table 4. Strain A or A.SW skin grafts did not survive permanently in any of 135 (A × A.SW)F_2 recipients. When ovaries were grafted instead of skin, 5 out of 73 or 6.8 percent of the (A × A.SW)F_2 hybrids tolerated strain A ovaries for more than 150 days and 7 out of 66 (10.6%) of the (A × A.SW)F_2 hybrids tolerated A.SW grafts. Also, the distribution of survival times for ovarian grafts ultimately rejected was shifted to the right as compared to skin grafts (*cf.* text figs. 1, 2, 3,

TABLE 2.—Survival time in days of A, A.SW, A.BY, and A.CA skin grafts to F_2 hybrid recipients

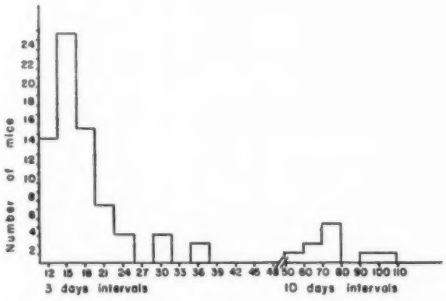
Expt. No.	Donor	Recipient	Survival (days) 11-13	14-16	17-19	20-22	23-25	26-28	29-31	32-34	35-37	38-40	41-43	44-46	47-49	50-59	60-69	70-79	80-89	90-99	100-120	121-150	>150	Percent permanent survivors	Total number of mice	Mean survival time of rejected grafts ± SE
1	A	(A × A.SW)F_2	13	24	14	6	3		3		2					1	2	4		1	1				74	24.6 ± 1.6
2	A.SW	(A × A.SW)F_2	22	19	19	5	3	2	1			3		3		1	2	2		1					83	22.2 ± 1.7
3	A	(A × A.BY)F_2		13	10		4	6	2	8	3	4	1	3	4	4	3		2		2		7	9.2	76	33.7 ± 2.4
4	A.BY	(A × A.BY)F_2		10	2		6	4	9	1	3	3	3	5	2		3	2	4	1	4	1	4	6.0	67	28.1 ± 3.5
5	A	(A × A.CA)F_2		7	8	3	7	5	4	5	6	5	2	2	3	3	4	1			3		3	4.2	71	35.8 ± 2.5
6	A.CA	(A × A.CA)F_2	2	4	9	6	5	5	9	3	6	4	1	2		2	2	2			2		2	3.1	65	32.9 ± 2.5
7	A.SW	(A.SW × A.BY)F_2	2	17	18	10	9	4	3	4	1	2	2							1					73	22.4 ± 1.3
8	A.BY	(A.SW × A.BY)F_2	2	20	15	9	5	5	6	5		1	2				2								72	22.7 ± 1.2
9	A.SW	(A.SW × A.CA)F_2	4	12	10	9	8	4	5	4	3	1	1												61	22.5 ± 0.9
10	A.CA	(A.SW × A.CA)F_2	4	7	9	9	4		3	3	1	2	1	3	4	2	3	3							58	31.1 ± 2.3
11	A.BY	(A.CA × A.BY)F_2	2	16	7	6	5	5	3	3						6	9	1	1	2	4		1	1.4	71	36.0 ± 3.2
12	A.CA	(A.CA × A.BY)F_2	3	17	5	9	3	4	3	5	6	3	1	2							3	4	1	1.4	69	33.6 ± 3.7

TABLE 3.—Comparison between the survival times in days of skin and ovarian grafts

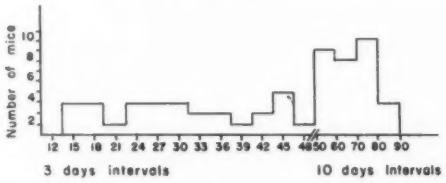
Expt. No.	Donor	Recipient	Skin grafts: Mean survival time ± SE	Skin grafts: Permanent survivors*	Skin grafts: Number of mice	Ovarian grafts: Mean survival time ± SE	Ovarian grafts: Permanent survivors*	Ovarian grafts: Number of mice
1	A	(A × C57L)F_1	—	10	10	—	10	10
2	A.BY	(A × C57BL)F_1	43.5 ± 2.8	None	20	—	20	20 (+2)†
3	(A.SW × A.BY)F_1	(A.SW × A.BY)F_1	26.2 ± 1.9	None	29	—	6	6
4	A.SW	(A.SW × A.CA)F_1	26.6 ± 4.9	None	10	—	3	3
5	A.SW	(A × A.SW)F_1	25.7 ± 5.7	None	7	83.6 ± 7.2	None	5

*The observation period was 150 days.
†One mouse died 40 days after transplantation while it still had a functioning graft and 1 never came into estrus, probably because of technical failure.

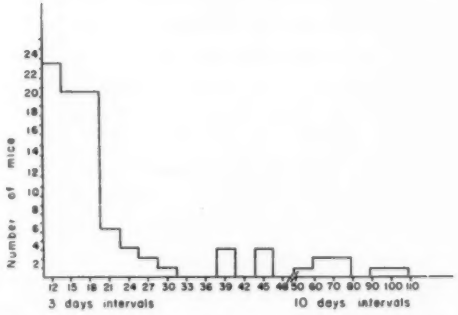
and 4). All permanent survivals were found in the *H-2* compatible combinations. Among 31 recipients that never came into estrus after ovarian transplantation, 25 were grafted with ovaries from *H-2* incompatible donors.



TEXT-FIGURE 1.—Survival of strain A skin grafts on (A × A.SW)F_2 hybrid mice.

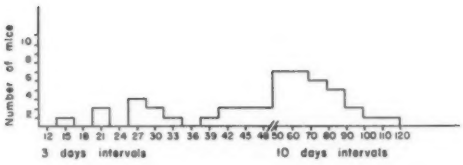


TEXT-FIGURE 2.—Survival of strain A ovaries grafted to (A × A.SW)F_2 hybrid mice as judged by the functional test. [*Note:* Recipients that never came into estrus after transplantation (13 animals) and those which accepted the ovarian grafts permanently (5 animals) were excluded from the histogram.]



TEXT-FIGURE 3.—Survival of strain A.SW skin grafts on (A × A.SW)F_2 hybrid mice.

Microscopic examination.—Postmortem examination to detect ovarian grafts was performed on 149 of 180 mice. Among those 149 recipients, 82 showed typical ovarian grafts or at least suspected scar tissue and they were therefore the subjects of further microscopical examination. The



TEXT-FIGURE 4.—Survival of strain A.SW ovaries grafted to (A × A.SW)F_2 hybrid mice as judged by the functional test. [*Note:* Recipients that never came into estrus after transplantation (18 animals) and those which accepted the ovarian grafts permanently (7 animals) were excluded from the histogram.]

TABLE 4.—Comparison between survival, in days, of skin and ovarian grafts

		Skin grafts		Ovarian grafts			
Donor	Serological type of F_2 recipients*	Mean survival time ± SE	Number of mice	Mean survival time of grafts rejected in more than 9 but less than 150 days ± SE	Survival for more than 150 days	Survival probably less than 10 days †	Number of mice
A	*H-2*ᵃ*H-2*ᵃ	29.4 ± 4.9	18	49.8 ± 3.6	4	1	20
A	*H-2*ᵃ*H-2*ᵇ	29.3 ± 4.9	30	48.6 ± 2.2	1	2	36
A	*H-2*ᵇ*H-2*ᵇ	15.0 ± 0.6	15	20.7 ± 1.0		10	17
A.SW	*H-2*ᵃ*H-2*ᵃ	14.7 ± 0.5	17	24.0 ± 1.1		15	21
A.SW	*H-2*ᵃ*H-2*ᵇ	27.2 ± 3.3	38	63.6 ± 3.5	5	2	35
A.SW	*H-2*ᵇ*H-2*ᵇ	20.6 ± 2.3	17	82.5 ± 4.6	2	1	10

*(A × A.SW)F_2 hybrids, divided into groups according to their serologically determined *H-2* type.

†As day of rejection could not be determined by the functional test before the 10th day after transplantation, figures of this column were excluded from the mean survival calculations.

result was in good agreement with the functional test, as judged by the vaginal-smear method. In animals with estrogenic activity (51 of 82 recipients examined) it was always possible to find a histologically well-preserved ovarium whereas not even remnants of organized ovarian tissue could be found with certainty in animals with no estrogenic activity. In some, fragments of fibrous scar tissue of unknown origin were seen. Those ovaries that did survive for 150 days according to the functional test showed a number of striking histological differences, related to the type of donor-host combination (table 5). Especially bad vascularization and lymphocytic infiltration of the ovarian graft could be observed in some combinations in which the ovaries survived in spite of weak (*non-H-2*) histoincompatibility between donor and host. This is in agreement with the findings of Harris and Eakin in their experiments on rats (*16*). Ovaries derived from A or A.SW mice and surviving on *H-2*-compatible (A × A.SW)F_2 hybrids (expts. 5 and 6) showed that the vascularization was poor in 12 mice (vascular congestion occurred in 10 and ischemia with necroses in 2) and all were more or less infiltrated with lymphocytes. In addition, 2 strain A and 3 A.SW grafts showed clear signs of fibrosis. In the combinations A.SW to (A.SW × A.CA)F_1, (A.SW × A.BY)F_1 to

TABLE 5.—Histological examination of functioning ovarian grafts 150 days after transplantation

Expt. No.	Donor	Recipient	Vascular disturbances*			Lymphocytic infiltration†			Number of mice
			−	+	++	−	+	++	
1	A	(A × C57L)F_1	10/10	0/10	0/10	10/10	0/10	0/10	10
2	A.SW	(A.SW × A.CA)F_1	3/3	0/3	0/3	2/3	1/3	0/3	3
3	(A.SW × A.BY)F_1	(A.SW × A.BY)F_1	5/6	1/6	0/6	2/6	4/6	0/6	6
4	A.BY	(A × C57BL)F_1	19/20	1/20	0/20	14/20	6/20	0/20	20
5	A	(A × A.SW)F_2	0/5	4/5	1/5	0/5	1/5	4/5	5
6	A.SW	(A × A.SW)F_2	0/7	6/7	1/7	0/7	2/7	5/7	7

*No detectable disturbance: −; vascular congestion: +; ischemia with necroses: ++.
†No infiltration: −; local infiltration around the blood vessels: +; diffuse infiltration: ++.

(A.SW × A.BY)F_1 and A.BY to (A × C57BL)F_1 (expts. 2, 3, and 4), similar pathological signs could be seen, though to lesser extent. There was no vascular damage or inflammation in the fully histocompatible system in which strain A ovaries were transplanted to (A × C57L)F_1 hybrids (expt. 1).

All functioning ovaries contained primordial and larger follicles, eggs, and corpora lutea. In all groups about 80 percent of the histologically examined ovaries were cystic, which lead to an increase of 30 to 40 times the original weight in some animals (fig. 1). The present findings were in agreement with Parkes (*23*) who found that long-term ovarian autografts and homografts in rats often were highly cystic and appeared "to be little more than relatively huge bags of fluid." In the donor material we found formation of ovarian cysts in 3 percent of 3- to 4-month-old mice, but this figure rose to 15 percent when 8-month-old mice were examined in a control material consisting of 40 A.BY mice. An induction of ovarian tumors by subtotal castration or "ovarian fragmentation" as reported by Lipschütz (*24*) was not observed in the present experiments nor was such a malignant development seen by Jones and Krohn (*25*) who sometimes found fluid-filled cysts in orthotopic (A, CBA, C3H, and RIII) mouse ovarian autografts after 25 days. The cysts were supposed to have arisen from the germinal epithelium. Similarly, after a mean observation period of 19.9 months, Marchant (*26*) found that, among the first-generation hybrid mice from C57BL mothers and IF fathers which were grafted with normal, compatible ovaries, 5 of 10 mice had macroscopic fluid-filled cysts and 3 more had unilateral ones. All attempts to detect tumor formation were in vain. When, as in our own experiments, A.BY ovaries were grafted to (A × C57BL)F_1 hybrids, 5 of 20 animals showed enlarged hemorrhagic follicles, but they had not developed what Lipschütz termed "luteomatosis."

Skin Transplantation to Mice Previously Grafted With Ovaries

Donor-host combination with weak histocompatibility differences.—It appeared from the previous sections that while grafted skin did not survive in the presence of weak (*non-H-2*) histocompatibility differences, grafted ovaries sometimes did. The question arose whether mice carrying such slightly incompatible, but nevertheless surviving and functioning, ovarian grafts would more likely tolerate subsequent grafting of skin from the same type of donor than recipients previously not exposed to ovarian grafts. This possibility was investigated by skin grafting 100 days after ovarian transplantation in the genotype combinations shown in table 6. The ovarian donors had been earmarked in the same way as their recipients and were saved for later skin transplantation. In all groups in which *H-2* differences were not involved there was a number of ovarian grafts surviving at this time. None were rejected during the subsequent observation period of 150 days, irrespective of the fate of the subsequent skin graft.

Among 41 skin grafts transplanted to mice bearing surviving ovarian grafts from the same donor type, 33 survived permanently (table 6, IIb),

TABLE 6.—Results of skin grafting to recipients previously grafted with ovaries from the same donor type and to untreated controls. Experiments 1, 2, 3, 4, 5, 8, and 9 involve weak (*non-H-2*) differences in histocompatibility, while in experiments 6 and 7 there is an additional *H-2* difference

				II. Recipients grafted with ovaries 100 days prior to skin grafting							
				I. Previously untreated recipients		(*a*) Ovaries rejected: results of skin grafting 100 days after ovarian grafting				(*b*) Ovaries surviving at 100 days: results of skin grafting 100 days after ovarian grafting		
Expt. No.	Donor	Recipient	Serological type of the F_2 recipient	Mean survival time ± SE (days)	Permanent survivors (>150 days)	Number of mice	Mean survival time of ovary ± SE (days)	Number of permanent survivors (>150 days)	Mean survival time ± SE of rejected skin (days)	Number of mice	Number of permanent survivors (>150 days)	Mean survival time ± SE of rejected skin (days)
1	A.BY	(A × C57BL)F_1		43.5 ± 2.8	0/20	0	—	—	—	20	15	26.0±3.2
2	(A.SW × A.BY)F_1	(A.SW × A.BY)F_1		26.2 ± 1.9	0/29	0	—	—	—	6	5	*
3	A.SW	(A.SW × A.CA)F_1		26.6 ± 4.9	0/10	0	—	—	—	3	2	†
4	A	(A × A.SW)F_2	*H-2^{a}H-2^a*	29.4 ± 4.9	0/18	16	49.8 ± 3.6	1	28.1 ± 5.0	4	4	—
5	A	(A × A.SW)F_2	*H-2^{a}H-2^s*	29.3 ± 4.9	0/30	35	48.6 ± 2.2	2	24.4 ± 2.9	1	1	—
6	A	(A × A.SW)F_2	*H-2^{s}H-2^s*	15.0 ± 0.6	0/15	17	20.7 ± 1.0	None	13.6 ± 0.8	0	—	—
7	A.SW	(A × A.SW)F_2	*H-2^{a}H-2^a*	14.7 ± 0.5	0/17	21	24.0 ± 1.1	None	13.2 ± 0.7	0	—	—
8	A.SW	(A × A.SW)F_2	*H-2^{a}H-2^s*	27.2 ± 3.3	0/38	30	63.6 ± 3.5	None	23.5 ± 3.1	5	5	—
9	A.SW	(A × A.SW)F_2	*H-2^{s}H-2^s*	20.6 ± 2.3	0/17	10	82.5 ± 4.6	1	48.3 ± 12.0	2	1	‡

*One recipient rejected the skin transplant 46 days after graft.
†One recipient rejected the skin transplant 27 days after graft.
‡One recipient rejected the skin transplant 44 days after graft.

in contrast to the experiments involving skin grafting to previously untreated recipients of the same genotype in which all grafts were rejected. Surprisingly enough, 4 skin grafts among 91 showed permanent survival across *non-H-2* differences after transplantation to recipients that had received ovarian grafts previously from the same genotype but rejected them prior to skin grafting according to the functional test (table 6, IIa).

Donor-host combination with strong histocompatibility differences.—When, on the other hand, a donor-host combination was chosen in which the strong *H-2* difference was involved, the grafted ovarium (which, according to the section "Isografts and homografts," was probably destroyed rather soon, at all events before it had started to function) had no suppressing effect on the homograft reaction against a subsequently grafted skin transplant (table 6, IIa). In fact, the transplanted ovaries had an immunizing effect against subsequent skin grafting. This was most clearly demonstrated in an experiment in which 5 spayed and 5 untreated A.SW mice were grafted with strain A ovaries 10 days before they were grafted with skin from the same donor strain. All skin transplants in both groups were violently rejected before the 9th day, which indicated a second-set reaction.

Transplantation of Neoplastic Tissue

In the previous study (*4*), tumor transplantation appeared to behave in strict accordance with the expectation that A and A.SW differed only with regard to a single histocompatibility factor, but skin grafting showed that this was not so. Large tumor inoculums were used throughout those experiments. When the (A × A.SW)F_2 recipients were preimmunized by a single inoculation of mixed kidney, liver, and spleen cells of A.SW origin 7 days before tumor inoculation, the mammary carcinoma S3W gave the same high percentage of takes, 81, as in untreated hybrids. The same was true when the F_2 mice were pretreated with strain A tissue and subsequently challenged with the mammary carcinoma S10S of strain A origin. Thus large tumor grafts in untreated or preimmunized mice failed to reveal the numerous weak histocompatibility factors that were segregating in the A × A.SW cross. Neither did they reveal the residual heterozygosis of the 3 IR lines A.SW, A.BY, and A.CA. A renewed attempt was made in the present study to demonstrate some of the weak barriers using *small* inoculums and preimmunized hosts.

Lymphoma SL1 of strain A origin, and lymphoma WL*4*, carcinoma S3W, and sarcoma MWE of A.SW origin were first tested in untreated A, A.SW, A.BY, and A.CA hosts with the usual large inoculums. All tumors grew well within the original, *H-2* compatible strain, but refused to transgress *H-2* barriers (table 7).

In the first series of the preimmunization tests, thymocytes (*27*) were used for pretreatment. In the cell dose (10^7) and according to the schedule found optimal by Snell (*27*), they were inoculated intraperitoneally 11 days before tumor grafting in a single dose into 2 groups of (A × A.SW)F_2 hybrids containing 17 animals each, one group receiving A.SW

TABLE 7.—Summary of tumor transplantation tests

Tumor	Type	Genotype of origin	Progressively growing tumors in			
			A	A.SW	A.BY	A.CA
SL1	Lymphoma	A	21/23	0/29	0/6	0/4
WL4	Lymphoma	A.SW	0/10	35/37	0/10	0/4
S3W	Carcinoma	A.SW	0/15	25/25	0/16	0/16
MWE	Sarcoma	A.SW	0/24	34/35	0/23	0/16

cells and the other A cells. Two control groups of the same size were left untreated. After 11 days, 5×10^5 SL1 cells were inoculated subcutaneously into one control group and the group preimmunized with A cells, while the remaining two groups received 5×10^5 MWE cells. Of the mice grafted with SL1, 4 of 17 pretreated animals and 7 of 17 controls developed tumors. The corresponding figures for MWE were 10 of 17 and 12 of 17, respectively. The differences are slight and their significance is doubtful.

According to Medawar *et al.* (*28*), skin grafts have a good immunizing capacity, and very thin skin homografts, 1½ mm², are already able to induce maximal second-set response against subsequent skin grafting. For this reason, skin grafts were used for preimmunization in the second series of experiments and, to eliminate *H-2* segregation, (A.SW × A.BY)F_1 hybrid mice received small tumor grafts of A.SW origin. All animals were of the same age (about 4 months), randomly selected from the same colony. Twenty-nine mice had been pretreated with skin grafts from (A.SW × A.BY)F_1 donors—the donors and recipients always the same sex. The skin transplant was about 15 mm². In agreement with the previous findings, all recipients except one rejected the grafted skin after an average survival of 26.2 days. Fifty days after the skin transplantation the mice were challenged with various cell doses from the 3 A.SW tumors.

From experiments I and II in table 8 it can be seen that the weak histoincompatibility can be revealed by the use of small cell doses and preimmunized mice. Thus, 2×10^5 MWE sarcoma cells grew in all control and preimmunized mice after a short latent period (about 10 days in both groups). With 2×10^4 cells inoculated, however, 6 of 7 preimmunized animals failed to develop tumors while 5 of 7 controls succumbed with progressively growing neoplasms.

In experiment III, 10^5 WL4 lymphoma cells were grafted. They were rejected in all preimmunized recipients but grew and killed all untreated mice. With 2×10^4 S3W carcinoma cells (expt. IV), there was no significant difference between the pretreated animals and the controls, the number of takes being 3 of 8 and 2 of 6, respectively. from the data shown in table 8, no correlation can be established between the survival time of the skin grafts used for preimmunization and the ability of the recipient to reject a subsequent tumor graft. All tumors that reached a palpable size continued to grow progressively and ultimately killed the hosts.

TABLE 8.—Summary of tumor transplantation tests with known small cell doses in untreated and preimmunized (A.SW × A.BY)F_1 hybrid mice

Mice preimmunized with (A.SW × A.BY)F_1 skin				Untreated mice	
Mouse No.	Type of tumor and cell dose inoculated	Survival time of skin grafts from (A.SW × A.BY)F_1 mice (days)	Latent period after inoculation until tumor appeared (days)	Mouse No.	Latent period after inoculation until tumor appeared (days)
Expt. I	MWE, 2×10^5			Expt. I	
1		12	10	30	7
2		22	10	31	9
3		22	10	32	10
4		22	10	33	10
5		23	12	34	10
6		24	12	35	10
7		24	10	36	10
8		24	7	37	10
Expt. II	MWE, 2×10^4			Expt. II	
9		14	29	38	18
10		23	No take	39	19
11		23	"	40	19
12		26	"	41	22
13		30	"	42	36
14		32	"	43	No take
15		36	"	44	"
Expt. III	WL4, 10^5			Expt. III	
16		21	No take	45	6
17		23	"	46	7
18		23	"	47	10
19		23	"	48	10
20		26	"	49	12
21		37	"	50	33
Expt. IV	S3W, 2×10^4			Expt. IV	
22		17	No take	51	22
23		20	"	52	23
24		21	46	53	No take
25		25	34	54	"
26		30	No take	55	"
27		45	"	56	"
28		65	"	57	†
29		*	35	58	†

*Animal was still carrying its transplant at its death 120 days after skin grafting.
†Animal died of unknown cause 1st week after tumor grafting.

DISCUSSION

A previous study involving skin grafting within some of the "coisogenic resistant" (IR) lines of Snell (*6*) with a strain A background and their F_2 hybrids has shown that lines A and A.SW do not fulfill the theoretical expectation that they differ only with regard to a single histocompatibility factor. It also became clear that the sublines A.BY, A.SW, and A.CA were not homozygous. Further skin-grafting tests performed in the present study clearly showed that lines A.BY and A.CA also differed from the A strain with regard to several histocompatibility factors. After the previous paper had been published, Dr. G. D. Snell traced the origin of these striking deviations from the theoretical expectation to a contamination of the A strain used for his backcrosses during the early history of lines A.BY, A.SW, and A.CA (*8*). Other coisogenic resistant

lines of Snell seem to fulfill the postulated one-gene difference much better (*29*). The lines used in the present study appear therefore exceptional and, since the contamination seems to be the explanation, their case is rather trivial. It was nevertheless worth while to use these strains and their segregating hybrids as a tool in further transplantation studies. This was motivated by the previous finding (*4*) that, while skin grafts of A and A.SW origin were uniformly rejected if grafted to *H-2* compatible F_2 hybrids that were segregating with regard to some of the weak (*non-H-2*) histocompatibility factors that separate A and A.SW, tumor grafts were equally regularly accepted and even preimmunization was unable to tip the balance in favor of the host. Comparatively large tumor-cell doses were used for inoculation. In the present work, it was possible to demonstrate some weak barriers in question even with tumors, but only under rather critical conditions where preimmunization was used in combination with the inoculation of small tumor-cell doses.

The discrepancy between the transplantation behavior of skin and tumor grafts is by no means surprising, since many previous findings indicate that skin transplants are very sensitive to weak histocompatibility differences while tumor grafts as a rule easily transgress them. This is understandable because skin is a very exacting tissue with regard to graft bed, vascularization, etc. Furthermore it tends to maintain *status quo* after orthotopical transplantation. Contrariwise, grafted tumors tend to proliferate progressively and may be less exacting with regard to vascularization requirements; weak or slow homograft reactions may never catch up with the increasing mass of the graft. These differences are usually assumed to explain the discrepancy between the grafting behavior of skin and tumors. It may be questioned, however, whether this is the whole explanation. A recent paper of Winn, Stevens, and Snell (*30*) indicates, for example, that certain differences at the histocompatibility-1 (*H-1*) locus tend to influence the behavior of tumor grafts in preference to skin grafts, in contrast to the usual situation.

These considerations motivated a search for some normal tissue that does not grow progressively as a transplanted tumor but might nevertheless be less vulnerable to weak homograft reactions than skin transplants. As a first choice, ovarian grafting was attempted, partly because of the ease with which the survival of ovarian grafts can be assessed in castrated females by a functional test, and partly because there are numerous papers in the previous literature which suggest that ovaries might sometimes survive in spite of various degrees of histoincompatibility. This rather controversial field has recently been reviewed critically by Krohn (*31*) who has contributed greatly to the clarification of the issues involved in in his own experiments. Krohn and coworkers are of the opinion that their findings do not indicate any basic difference between the behavior of ovarian and nonendocrine grafts. This was most obvious when mice were used as experimental animals and the histocompatibility barrier included the strong *H-2* locus. In such systems, ovarian grafts were fully capable of provoking a homograft reaction and were as vulnerable

to it as skin grafts. The degree of immunity induced was sufficient to cause rejection of a subsequent skin graft just as if the first graft had been skin rather than ovarian. On the other hand, Hicken and Krohn (*10*) noticed that second-set ovarian grafts transplanted from a parental strain to F_2 hosts, which destroyed a first ovarian graft only after an extended period of acceptance, did not necessarily undergo an accelerated second-set breakdown, and sometimes survived for much longer periods than the first graft. They have discussed a possible relationship between this finding and immunological enhancement.

When comparing the survival of strain CBA and strain A skin and ovarian grafts on (A × CBA)F_2 hybrids, Hicken and Krohn (*10*) *did* find a certain difference, showing a higher percentage of surviving ovarian than skin transplants. They attributed this to random differences in the genetic segregations of the two sets of F_2 hybrids. This view was mostly based on the finding that the mice in an F_2 cross, which accepted ovarian grafts from one parental strain, often also accepted a skin graft from the same strain transplanted on a later occasion. It was assumed that compatible mice had been fortuitously selected from the total F_2 population that tolerated both ovarian and skin grafts. The success of the second test graft with skin was taken as a confirmation of compatibility. The possibility that the successful first ovarian graft could have permanently impaired the capacity of the host to respond to further grafts was suggested but dismissed.

The present study fully confirmed the view of Krohn that ovarian and skin grafts do not differ in their immunological requirements as long as the *H-2* barrier is considered. In contrast, there was a striking difference between the ability of the two tissues to survive when confronted with some of the *non-H-2* histocompatibility factors segregating in the mice used. Ovarian grafts showed a much better survival, and the differences between the two tissues were of such an order of magnitude and of such regularity that possible unconscious selection of fortuitously compatible animals can be confidently excluded. For example, 29 (A.SW × A.BY)F_1 hybrids rejected skin grafts from (A.SW × A.BY)F_1 hybrids while ovarian grafts permanently survived in 6 out of 6. As another example, (A × C57BL)F_1 hybrids (*H-2*^{a}*H-2*^{b}) rejected A.BY skin grafts (*H-2*^{b}*H-2*^{b}) in all 20 animals while 20 of 20 ovarian transplants survived in the same combination during the entire observation period of 150 days. Moreover, a number of animals of different genotypes carrying ovarian grafts surviving in spite of *non-H-2* barriers also accepted skin transplants in several cases from the donor genotype of the ovaries at a later date, though they would have uniformly rejected such skin grafts if previously untreated. This surprising finding may also serve as a possible explanation, instead of selection of the results of Krohn regarding the unexpectedly high survival rate of ovarian and subsequent skin grafts in his F_2 cross. In a few cases of the present study, skin that was grafted after ovarian transplants actually survived in spite of the fact that the functional test indicated that the ovarian graft had been rejected. This was an exception, however,

and might possibly have been due to the survival of some nonfunctional part of the ovarian tissue which was capable of maintaining the condition necessary for the survival of the skin graft.

The data indicate that the survival of the grafted ovaries was not due to an absence of the homograft reaction. Histological examination showed that the surviving ovaries were different from autografted or isografted ovarian tissue in many instances, since they were infiltrated with lymphocytes and other round cells and showed various degenerative changes. Moreover, even if the ovaries would have failed to immunize the recipients, the subsequently applied skin graft would undoubtedly have done so and would have elicited a homograft reaction against itself and the ovary as well. Thus the finding cannot be attributed to the lack of antigenic stimulus.

Special attention was paid to the question whether tumor formation occurred in the grafted ovarium or not. In agreement with data from other laboratories (*25*, *26*), macro- and microscopic examination of surviving grafts showed a considerably increased size due to a cystic transformation, but in spite of careful histological examination, there were no signs of malignancy in the grafts.

The question arises whether the changes in the hormone balance of a castrated animal may possibly be held responsible for the surprisingly good survival of the grafted ovary across the *non-H-2* barriers. This has been postulated to explain the results of most previous authors (*16*, *32*), which indicated a certain homotransplantability of ovarian tissues. As recently shown by Lamond (*33*) there is at present no evidence that exogenous gonadotrophin administration in any way interferes with the homograft reaction in mice, though it seems to stimulate the estrogen production in the early history of the graft. The importance of an adequate hormonal environment or the "need" for the gland's hormones cannot be decided on the basis of the present experiments which did not involve ovarian grafting to intact females, but it can be argued that hormone stimulation alone could hardly explain the survival of subsequently grafted skin.

It is difficult to escape the conclusion that the grafted ovaries induced a changed condition in the host which resulted in the acceptance of both ovarian and skin grafts, provided that the antigenic difference was not too large. Whether this phenomenon is akin to induced tolerance, immunological paralysis, or immunological enhancement cannot be subjected to discussion at the present time, but should be the object of further detailed study.

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PLATES

PLATE 42

FIGURE 1.—An (A × C57L)F_1 hybrid 150 days after grafting of 2 ovarian halves from a strain A mouse. Highly cystic grafts are invaded by fairly large branches from the femoral arteries and their weights have increased about 30 times since day of transplantation.

597373—61——13

Induction of Epidermoid Carcinoma in Lungs of Rats: A "New" Method Based Upon Deposition of Methylcholanthrene in Areas of Pulmonary Infarction [1]

MEARL F. STANTON *and* ROBERT BLACKWELL,
Laboratory of Pathology, National Cancer Institute,[2]
Bethesda, Maryland

SUMMARY

Multiple pulmonary infarcts were induced in rats by intravenous injection of the halogenated hydrocarbon, hexachlorotetrafluorobutane. When 3-methylcholanthrene (MCA) was mixed with this infarct-inducing vehicle and injected, the resultant infarcts were the sites of invasive epidermoid carcinomas. Transitional lesions in the infarcts indicated that the carcinomas were derived from regenerating epithelium frequently converted to keratinized squamous epithelium by the carcinogen. The tumors are comparable in their morphology and behavior to the bronchogenic carcinoma of man and may serve as a prototype for the study of this disease. In control experiments we used emboli of MCA mixed with tricaprylin. This mixture did not infarct the lung but resulted in the development of massive keratinized cysts that did not become invasive carcinomas. However, when rats were treated similarly with emboli of MCA and the lungs were later infarcted with the fluorocarbon alone, the cysts developed, squamous metaplasia was present in some healed infarcts, and two carcinomas were found. The carcinomas seemed to originate at sites of infarction. These observations suggest that the process of infarction and repair may determine the malignant behavior of the tumors. It seems reasonable that physiological changes at the site of infarction, such as ischemia and regeneration, play a major role in this process. The procedure offers a simple and efficient means of introducing and testing other potential carcinogens in the lung, since it is evident that carcinomas of the lung can be readily induced by a pulmonary carcinogen, if the carcinogen is deposited in lung tissue that is damaged or destined to become damaged through infarction.—J. Nat. Cancer Inst. 27: 375–407, 1961.

THROUGH DIFFERENT methods of administration, a variety of substances are capable of inducing epithelial tumors of the lung in rodents (*1–21*). However, few of these methods induce pulmonary tumors of a type and behavior that simulate the bronchogenic carcinoma of man. Furthermore, unless radioactive material are used as the carcinogen, none of these methods yield a sufficiently high incidence of bronchogenic carcinoma to merit use as a method of detecting pulmonary carcinogens of

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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relatively weak potency. In tests of potential carcinogens, it would be an advantage to insure that the substance used is deposited securely near pulmonary epithelium and permitted to act when lung tissue is most susceptible to tumor induction. One means of achieving this advantage may be to deposit the carcinogen in areas of pulmonary damage, so that the carcinogen is exposed to tissues undergoing repair and regeneration.

We wished first to test this hypothesis by depositing 3-MCA, a known carcinogen for rodent lung, in areas of pulmonary tissue simultaneously or subsequently damaged through infarction. The lung was infarcted by the intravenous injection of hexachlorotetrafluorobutane, a liquid halogenated hydrocarbon that obstructs pulmonary blood vessels of capillary size (*22*). The areas of infarction become ischemic and remain so from the moment the fluorocarbon reaches the capillary bed. Rapid healing by fibrosis follows infarction and during this time marked regeneration of epithelium occurs within and at the margins of the infarcts. Thus, the fluorocarbon and whatever carcinogen might be added to it are trapped in the area of infarction and probably remain in the area during the time when epithelial regeneration occurs. The following experiments show the oncogenic effect of infarcting the lung with a mixture of 3-MCA and fluorocarbon, the oncogenic effect of simple emboli of 3-MCA, and particularly the differences in the behavior of tumors that occur when infarction of the lung follows embolization of the lung with 3-MCA.

MATERIALS AND METHODS

Animals.—The rats were of the Osborne-Mendel strain of a colony maintained by the Animal Production Section of the National Institutes of Health. They were of either sex, 5 to 7 months old, and weighed 300 to 350 g at the time of injection. The rats were housed in suspended cages of wire and were fed commercially prepared rodent pellets.

Several diseases of the lung that may be related to the lesions induced in these experiments occur spontaneously. Rats of many strains, including OM/N, commonly develop a slowly progressive pneumonia that is presumably of viral origin (*23, 24*). As a consequence of this disease, at least 80 percent of the rats of our colony has some degree of pulmonary inflammation at 12 months of age (*25*); more will be said later of this disease. The incidence of spontaneous epidermoid carcinoma of the lung in rats is remarkably low (*25–29*), even though many carcinogens are capable of inducing this type of tumor (*9–21*). Spontaneous pulmonary tumors of the alveologenic type have been described (*30*), and Snell has observed 5 spontaneous tumors of this type in rats over 12 months of age from our colony (*25*). Alveologenic tumors differ in their morphology from the tumors described in this report and were not observed in our experimental animals. The known susceptibility of rats to polyoma virus (*31*), and the report of Rabson *et al.* (*8*) that this virus can cause epithelial tumors of the lung in hamsters suggest that the polyoma virus

might contribute to the induction of the tumors reported here. At 8 weeks of age, the rats were housed in animal rooms that had contained mice infected with this virus. Consequently, air-borne contamination was possible. However, in other experiments, we have inoculated young rats with polyoma virus by a variety of methods, including the method described here, and have not induced lung tumors. Nevertheless, since pretreatment with MCA enhances the proliferative response to some tumor viruses (*32*), the possibility of an oncogenic virus contributing to the formation of the tumors cannot be excluded entirely.

Injections.—Hexachlorotetrafluorobutane (d^{25} 1.83, bp^{745} 204°) was obtained from the Dow Corning Corporation, Midland, Michigan. The compound is miscible with most organic solvents and tricaprylin. Tricaprylin was obtained from Eastman Kodak Company, Rochester, New York, and purified 3-MCA from Edcan Laboratories, South Norwalk, Connecticut.

All injections were made under ether anesthesia through 20-gauge needles directly into surgically exposed femoral veins. Each injection was 0.05 ml. This volume of fluorocarbon is sufficient to infarct approximately $\frac{1}{10}$ of the total lung tissue in a rat weighing 300 g. Infarcts of this extent are responsible for death of 1 rat in 5 within 14 days. Acute mortality from pulmonary infarction is increased if larger doses or smaller rats are used.

Method of study.—Some rats died or were killed shortly after injection and at intervals during the ensuing 12 months. With the exception of the rats receiving injections of fluorocarbon only, the rats that survived were killed within 15 months after injection. Complete autopsies were performed; lungs and any grossly abnormal organs were studied histologically. Tissues were fixed in 10 percent neutral formalin and routinely stained with hematoxylin and eosin. We fixed the lungs by filling through the trachea. Each lobe was sectioned in a plane through the hilus and the apex of a lateral angle at the pleural surface. Selected tissues were stained with Masson's trichrome, periodic acid-leukofuchsin, and Mayer's mucicarmine.

RESULTS

Rats Receiving Injections of Fluorocarbon Only (Controls, Table 1)

During the experiments, 62 rats received intravenous injections of 0.05 ml of hexachlorotetrafluorobutane. Over half were kept for more than 12 months after injections, and 12 for over 2 years. Infarcts or the scars of infarction were found in all but 6. The infarcts were multiple and were consistently at the pleural surface. The acute infarcts were pyramidal with the pleural surface forming a broad base and the deeper substance of the lung forming an irregular apex. Medium-sized vessels and bronchi were found frequently at this apex. The caudal half of the left lung was the most common site of infarction, this area being

involved in over two thirds of the rats. The progressive changes of ischemic necrosis, marginal inflammation, scarring, and proliferation of marginal epithelium were like the changes previously observed in the rabbit (*22*) (figs. 1 through 3). Infarcts over 70 days old were flat, irregular scars on the pleural surface that did not extend deep into lung tissue. These healed infarcts were free of acute inflammatory infiltrate and consisted of dense collagen sometimes infiltrated with mononuclear leukocytes and pigment-laden macrophages. The pleural surface of the scars showed no mesothelial proliferation. The characteristic epithelial components of the healed infarcts were microcysts of respiratory epithelium that had been trapped within fibrous tissue at the margins of the scar. The microcysts were lined by thin layers of cuboidal or columnar epithelium, in some areas clearly ciliated (fig. 2). The periodic acid-Schiff (PAS) and mucicarmine stains indicated that epithelial linings and lumens often contained mucin. A direct connection could be traced between some microcysts and respiratory passages. Dilated, distorted bronchioles were often pulled into the margins of the scars by contraction (fig. 3). The complex of minute epithelial cysts imbedded in focal scars along the surface of the lung was present in rats killed as long as 3 years after injection, but there was no evidence of progressive epithelial proliferation or squamous metaplasia in the infarcts.

Chronic murine pneumonia and its sequela, bronchiectasis, were present to some degree in many rats. This complex of destructive and inflammatory changes is easily distinguished from the lesions of infarction even when the two are in apposition. In murine pneumonia, extensive inflammatory lesions follow the distribution of the bronchial tree while the infarcts are characterized by wedge-shaped scars extending only short distances into the lung from wide bases at the pleural surface. The infarcts do not involve major bronchi primarily, while the lesions of chronic murine pneumonia are more severe in the main-stem bronchi that lie at the hilus of the lung.

Rats Receiving Injections of Fluorocarbon Containing Crystals of MCA (Expts. 1 and 2, Table 1)

In experiment 1, MCA crystals were suspended in the fluorocarbon and injected at a dose of approximately 2 mg of MCA in 0.05 ml of fluorocarbon. (The solubility of MCA in fluorocarbon is approximately 6 mg/ml at 40° C.) Each rat then received 0.3 mg of dissolved MCA plus approximately 1.7 mg of undissolved crystals. Although this mixture was agitated vigorously before each injection, the difference in the density of fluorocarbon (1.83) and undissolved MCA crystals (1.28) permitted some separation of vehicle and carcinogen. In the subsequent 2 experiments (2a and 2b), we achieved a more uniform suspension by using a mixture of 1 part tricaprylin to 3 parts fluorocarbon. The lower density of tricaprylin (0.954) brought the density of the mixture close enough to that of crystalline MCA to cause the crystals to remain dispersed in the

TABLE 1.—Incidence of squamous metaplasia in infarcts, epidermoid cysts, and carcinomas in lungs of rats after intravenous injections of MCA in hexachlorotetrafluorobutane and tricaprylin; table excludes rats that died within 30 days

Procedure				Totals				A quarterly account of: Carcinomas/epidermoid cysts/squamous metaplasia				
								Number of rats examined				
Expt. No.	Agent	Vehicle	Method	Number of rats	Carcinoma	Epidermoid cysts	Squamous metaplasia	2–4 months	5–7 months	8–10 months	11–13 months	>13 months
	Controls	Fluorocarbon 0.05 ml	Single intravenous (iv) injection	62	0	0	0	0/0/0 1	0/0/0 20	0/0/0 4	0/0/0 8	0/0/0 35
1	MCA: 2 mg crystals	Suspended in 0.05 ml fluorocarbon	" "	10	2 (20%)	2	2		0/0/0 1	0/1/0 1	2/1/2 8	
			" "									
2a	MCA: 2 mg crystals	Suspended in 0.05 ml 75% fluorocarbon 25% tricaprylin	Single iv injection	21	8 (38%)	12	8	0/0/0 2	3/3/3 4	3/2/3 3	2/5/1 7	0/2/1 5
2b	MCA: 2 mg crystals	" "	Repeated once 4 weeks after first injection	16	8 (50%)	5	12		3/2/9 11	4/3/3 4	1/0/0 1	
		" "										
3a	MCA: 2 mg crystals	Suspended in 0.05 ml tricaprylin	Single iv injection	20	0	9	0	0/1/0 1	0/3/0 4	0/3/0 5	0/1/0 9	0/1/0 1
3b	MCA: 2 mg crystals	" "	Followed in 4 weeks by 0.05 ml iv fluorocarbon	15	2 (13%)	6	4	0/0/0 1	1/2/2 2	1/1/1 5	0/1/0 2	0/2/1 5
		" "										
4a	MCA: 1 mg solution	Dissolved in 0.05 ml fluorocarbon at 70° C	Single iv injection	18	4 (22%)	0	6	0/0/1 2		2/0/2 3	2/0/2 6	0/0/1 7
4b	MCA: 1 mg solution	" "	Repeated once 4 weeks after first injection	16	7 (44%)	0	10	0/0/2 2	2/0/3 5	4/0/3 5	1/0/1 1	0/0/1 3
		" "										

mixture while the injections were done. The addition of tricaprylin to the fluorocarbon did not alter the capacity of the vehicle to cause infarction nor did it appreciably increase the amount of MCA that dissolved in the mixture over the amount dissolved in fluorocarbon alone. In experiment 2a, rats received intravenous injections of a single dose of 2 mg of MCA suspended in 0.05 ml of fluorocarbon-tricaprylin mixture, a dose comparable to that of MCA in experiment 1. In experiment 2b this dose was injected twice at intervals of 4 weeks.

The resultant lesions in the 2 experiments did not show qualitative differences, but varied only in the number of lesions and the number of rats affected.

More rats died shortly after injection with suspensions of crystalline MCA in fluorocarbon than with fluorocarbon alone. Presumably, the higher mortality resulted from embolism of large pulmonary vessels by MCA crystals, since, in addition to the infarcts noted in control rats, focal hemorrhagic areas were present throughout the lungs of rats that died early. Rats dying within the first 30 days often had multiple pulmonary abscesses.

The lungs had 3 types of proliferative epithelial lesions that were not present in the lungs of rats receiving injections of fluorocarbon alone. The 3 lesions, often in the same lung, consisted of squamous metaplasia of the regenerating epithelium in the scars of infarcts, epidermoid carcinomas, and massive epidermoid cysts.

The acute infarcts did not differ from those in rats receiving fluorocarbon only (fig. 4), but in many older infarcts there was a metaplastic conversion of the regenerating respiratory epithelium of the microcysts in the healed infarcts to a squamous epithelium. The microcysts were sometimes filled completely with squamous epithelial cells (figs. 5 and 6). The organization and differentiation of the epithelium were remarkably uniform. Stratification as well as keratinization was common and, with few exceptions (fig. 7), the epithelium of the microcysts did not have the disoriented structure characteristic of carcinoma *in situ*. In 13 rats, scars of infarction with microcysts lined by squamous epithelium merged with massive areas of proliferating keratinized epithelium (fig. 8). Some epithelial tissues within these macroscopic lesions were keratinized microcysts, while other areas of squamous epithelium did not have structural organization and seemed to invade adjacent fibrous tissue.

Eighteen rats in the 2 experiments had epithelial tumors that were unequivocally malignant (table 1) (fig. 14). Some areas in each tumor contained cells that formed keratin, but the cells varied in their degree of differentiation. Some were predominantly composed of well-differentiated squamous epithelium; their metastases also contained large cystic areas of keratin and epithelial pearls. Others were formed of invasive irregular tongues of bizarre epithelial cells in a sclerotic stroma or of solid cords and sheets of anaplastic epithelial cells; massive keratin deposits were found less frequently (figs. 16 through 19). Six of the carcinomas were composed chiefly of epithelial cells with an adenomatous growth

pattern (fig. 13). These primarily glandular carcinomas contained areas of intracytoplasmic and extracytoplasmic mucin similar to those observed in regenerating microcysts of the infarcts, but they also had isolated areas of keratinized epithelium. Variations of these types occurred within a single tumor and could easily have represented structural modulations of a single cell type (fig. 23). The variations were good prototypes of the more common patterns observed in human bronchogenic carcinoma. However, no tumors in this experiment or the experiments described later simulated the so-called oat-cell carcinoma or alveolar carcinoma of man.

The carcinomas metastasized to many organs. Twelve tumors metastasized to hilar lymph nodes (fig. 20), 7 to kidney (fig. 21), 4 to adrenals, 3 to heart, 3 to liver, 1 to distant nodes, and 1 to ovaries. Local invasion of vertebrae occurred in 10 tumors, 7 invaded the diaphragm, and 4 the mediastinum. Invasion of blood vessels and perineural lymphatics was common. Two malignant tumors that invaded vertebrae stimulated a marked osteoblastic reaction in vertebral periosteum (fig. 22).

The original site of some of the carcinomas was clearly a healed infarct; the carcinomas were often contiguous with squamous epithelial foci in infarct scars (figs. 10 and 12). Extensive involvement of the lung obscured the origin of far-advanced carcinomas, but there was some evidence that all had arisen in infarcts. The caudal half of the left lung (the most common site of infarction) was the primary site of 10. Many rats had healed infarcts with lesions of squamous epithelium that were intermediate between microscopic foci within the microcysts and squamous carcinomas that metastasized.

Malignant behavior of some tumors was indicated by their structural disorganization and the apparent immaturity and variability of the cells (fig. 16), but others that metastasized widely contained masses of keratin and were composed chiefly of cells well differentiated as squamous epithelium (figs. 20 and 22). It was difficult to separate by morphological evidence all epithelial lesions in the scars according to whether they were potentially malignant. Therefore, the term "squamous metaplasia" was applied to most of the lesions and *the term carcinoma was reserved for those tumors that had metastasized to distant organs or that clearly invaded structures adjacent to the lung.* Thus, any error in the prediction of the behavior of the tumors designated in the table can be presumed to be in the underestimation of their malignant potential.

The third type of epithelial lesion was essentially a keratinizing epidermoid cyst. Multiple lesions of this type ranging to 1 cm in diameter were found throughout the lungs of 15 rats with carcinomas and 4 without carcinomas. The cysts were composed of a circumferential wall of differentiated stratified squamous epithelium enclosing a large central mass of desquamated keratin. Layers of cells with keratohyalin granules and epithelial cells in late stages of cornification formed the inner wall of the cysts (fig. 27). Most cells in the wall were polygonal, but those of the outer layer were cuboidal and formed a distinct limiting margin. A few

cells in mitosis within the outer margin were the only indication of continued growth. This outer margin was sometimes sufficiently irregular to suggest indolent invasion of adjacent lung tissue, but the relatively thin walls and massive keratin deposits, even in the largest lesions, indicated that increase in size was primarily through expansion of the central cavity rather than through invasive extension.

The epidermoid cysts did not differ in cell structure from many large keratinized lesions that made up parts of the carcinomas, nor did they differ except in size from the microcysts lined with squamous epithelium within the infarcts.[3] A high degree of squamous differentiation was common to all three lesions. The principal feature that distinguished the epidermoid cysts from the carcinomas or the more overt "metaplastic" lesions in scars was their well-defined structural organization as simple cysts with a stratified wall. Their distribution was also unlike that of the carcinomas or the infarcts in that they were scattered at random throughout the lung. They had no predilection for the pleural surfaces, the scars of infarction, or the major bronchi (fig. 24). Nevertheless, the suspicion of invasion in a few indicated that epidermoid cysts might be the origin of some carcinomas.

Presumably, the epidermoid cysts unrelated to the infarcts were the result of emboli of MCA crystals lodged in large arterioles where collateral circulation prevented infarction. Experiment 3 was an attempt to determine whether emboli that contained an equal amount of MCA and did not cause infarction would result in the formation of the epidermoid cysts, and whether lesions of this type without the superimposed damage of infarction would become malignant.

Rats Receiving Injections of Tricaprylin Containing Crystals of MCA (Expt. 3, Table 1)

Tricaprylin was used exclusively as the vehicle for MCA because multiple pulmonary emboli without infarction result when it is injected intravenously. A single dose of 2 mg of MCA in 0.05 ml of tricaprylin was

[3] It is also important to distinguish the epidermoid cysts from cystic bronchiectatic cavities that may be lined by metaplastic epithelium. Passey *et al.* have noted that the latter can be confused with carcinoma of the lung (*33*). Bronchiectatic cavities usually can be identified by the structure of the walls or their connection with recognizable bronchi that are involved to a lesser degree (fig. 26). The cysts, however, are isolated, circumscribed nodules unrelated to damaged bronchi and sometimes are evident as lesions within or at the margins of pulmonary arterioles (fig. 25). The most distinct difference is the type of metaplastic epithelium that lines the cavities. The walls of the epidermoid cysts are composed mostly of a thick layer of keratinized squamous epithelium, and, since the cysts are not connected with bronchi, their lumens are filled with desquamated keratin (fig. 27). In contrast, bronchiectatic cavities rarely contain more than microscopic foci of keratinized epithelium and their lumens are filled with inspissated secretion or mucopurulent exudate. The paucity of keratinization of the metaplastic epithelium in murine bronchiectasis has been noted by both Passey *et al.* (*33*) and Innes *et al.* (*24*). Passey was convinced that extensive keratinization was the result of deficiencies in fat-soluble vitamins and succeeded in causing the lesions to keratinize by placing rats on deficient diets. In our experience with rats fed commercially prepared laboratory pellets, small areas of keratinization occur rarely, but the epithelial alterations generally consist of a diffuse hyperplasia that particularly affects the basal layers of epithelium. The wall of the diseased bronchus is relatively thin and composed of cells that do not differentiate completely (fig. 28). Extensive areas of poorly differentiated lining cells frequently become sufficiently distorted and disoriented to suggest neoplasia, but the low incidence of spontaneous bronchogenic carcinoma in the rat indicates that this change is unrelated to progressive tumor growth. This disorganized, anaplastic cell growth in bronchiectasis, a lesion that does not end as cancer, is a remarkable contrast to the complete differentiation of the cells in many carcinomas induced in this experiment.

injected intravenously in experiment 3a. The rats in experiment 3b were treated similarly, but 4 weeks later those surviving were given an intravenous injection of sufficient fluorocarbon to induce infarcts in the lung. MCA is nearly twice as soluble in tricaprylin as it is in the fluorocarbon or fluorocarbon-tricaprylin mixtures of the previous experiments. More dissolved MCA reached the lungs in this experiment than previously, but most of the MCA remained a suspension of crystals. The size of the crystals as measured with an ocular micrometer was the same in tricaprylin, fluorocarbon, and the fluorocarbon-tricaprylin vehicles. After passage through a 20-gauge needle, crystals were present in all 3 mixtures that varied in least diameter from less than 2 μ to over 30 μ.

Over half the rats died from pulmonary embolism within 30 days. The wedge-shaped, ischemic, peripheral infarcts characteristic of fluorocarbon injection were absent. The survivors of experiment 3a confirmed our assumption that large emboli containing MCA were responsible for the epidermoid cysts, because 9 of the 20 rats had lesions of this type (fig. 25). Furthermore, it was particularly significant that none of the 20 rats had malignant lung tumors. The cysts, if present, were multiple lesions. Many were as large as the previously observed carcinomas, and some had a multilocular structure in which the lining epithelium was in various stages of differentiation. In all the cysts, the massive deposition of keratin overshadowed epithelial cell proliferation and evidence of frank invasion was absent. Six of the 20 rats had areas of fibrosis within the lung which were of unknown etiology. The scars in 2 rats were adjacent to large keratinized cysts containing particularly bizarre epithelial cells, and distorted tongues of this disorganized epithelium extended for short distances into the scar at the margin of the cysts. These atypical changes could have resulted from distortion of the epithelium when the scar healed, but it was also possible that they represented early malignant behavior. The idea that lung damage and healing might influence the behavior of the epidermoid cysts stimulated us to attempt deliberately to damage the lung in the region of epidermoid cysts.

Experiment 3b was an attempt to induce scarring by infarction that would involve an area of the lung in which MCA had been deposited or epidermoid cysts were beginning to develop as a result of previous MCA emboli. The combined mortality for the 2 procedures was high, but 15 rats survived. Six of the 15 survivors had epidermoid cysts in the lung that were similar to previous cysts (fig. 24). However, 2 of the 6 rats also had a carcinoma of the lung. Each of the 2 carcinomas had invaded the chest wall and 1 had metastasized to the kidney. The criteria of invasion and metastasis were not necessary to distinguish the carcinomas from the epidermoid cysts. Both tumors were solid masses of disorganized anaplastic epithelial cells that formed keratin in some areas and in others were pseudo-glandular in structure. The extent of involvement made it difficult to determine whether either carcinoma had developed from an epidermoid cyst or an area of infarction. However, both tumors again involved the caudal half of the left lung, and at the margin of one (fig. 11)

an area of subpleural scarring and epithelial regeneration identical to that observed in infarcts was present. Three rats had healing infarcts in which squamous metaplasia was clearly evident. These areas of squamous metaplasia occurred in infarcts not adjacent to carcinomas or epidermoid cysts.

The final experiment, converse to the injection of crystalline emboli of MCA, was an attempt to exclude emboli of large size and deliver MCA to the lung in soluble form, particularly concentrated in areas of infarction.

Rats Receiving Injections of Heated Fluorocarbon Containing Dissolved MCA (Expt. 4, Table 1)

To inject dissolved MCA in amounts nearly comparable to those used in previous experiments, it was necessary to heat a mixture of MCA and fluorocarbon and keep it hot during the procedure. Only half the amount of MCA used previously would dissolve in fluorocarbon at 70° C (1 mg/0.05 ml), but recrystallization on cooling was slow enough to assume that all this would be carried particularly to the sites of infarction and perhaps in more concentrated amounts than in the previous experiments. The heated solution was used as a single injection in experiment 4a, and in experiment 4b rats received this solution twice with an interval of 4 weeks between injections. The experiment was comparable to experiment 2, but the total amount of MCA was only half as great and tricaprylin was not present in the mixture.

Acute mortality was no greater than that resulting from simple infarction and the acute infarcts were similar. The tumor response was different from that of experiment 2.

In experiment 4a, 4 of the 18 rats had carcinomas of the lung. In addition to these 4, two others had squamous metaplasia in healed infarcts. None had multiple epidermoid cysts, but 1 carcinoma extended from a large cystic keratinized tumor situated in the margin of a healed infarct. The extent of the carcinomas in the other 3 rats made it difficult to determine whether they had arisen in infarcts, but their apparent origin was the lower half of the left lung, the common site of infarcts. The 4 carcinomas were less differentiated than many of those in previous experiments. Desmoplasia was prominent, though the tumors were distinctly epithelial. The carcinomas had invaded vertebrae, ribs, pulmonary vessels, diaphragms, hearts, and hilar lymph nodes; no distant metastases were found.

In experiment 4b, all 16 rats had infarcts, showing in 10 extensive areas of keratinized epithelium. Three of the 10 had infarct scars in which microscopic vesicles lined with metaplastic keratinized epithelium were contiguous with grossly apparent, solid epithelial masses. Anaplasia and marginal areas suggestive of invasion indicated that these lesions probably represented transitions to overt carcinomas (fig. 9). An additional 7 rats had tumors that invaded structures adjacent to the lung and metastasized to either kidney, adrenal, or spleen. Only 4 of the 7 malig-

nant tumors were entirely comparable to the epidermoid carcinomas previously described (fig. 15). The other 3 were composed primarily of elongated spindle-shaped cells that lay in a collagenous matrix (fig. 29). Scattered among these cells were islands and syncytia of epithelial cells that were greatly distorted but still recognizable as epithelium. The tumors represented either a mixture of epithelial and mesenchymal cells or epithelial cells sufficiently undifferentiated to mimic sarcomatous structure (figs. 30 through 32). On morphological grounds, the latter seems the most reasonable explanation. However, it is possible that these represented tumors of both epithelial and mesenchymal cells, for tumors associated only with the lung in 3 rats were composed entirely of mesenchymal cells. These 3 "sarcomatous" tumors (not listed in table 1) were pedunculated masses attached to pleural surfaces at sites of infarction. They were composed of fascicles of uniform, spindle-shaped cells that did not invade the adjacent lung tissue.

In experiment 4b, the origin of the carcinomas and carcinosarcomas was no more obvious in every case than it was in the previous experiments, even though the multiple, massive epidermoid cysts could be eliminated as a source. Again, evidence pointed to the keratinized lesions in the infarct scars as the most likely source of the tumors. All malignant tumors involved the caudal half of the left lung, and 5 were contiguous with squamous epithelium within the scars of infarction. Three of the latter carcinomas seemed to arise from primary foci in the margins of scars in which large circumscribed masses of keratinized epithelium resembling the epidermoid cysts of the other experiments were present.

Primary Tumors in Organs Other Than Lung

Two carcinomas of the adrenal cortex were found, 1 in a male rat 13 months of age in the control group, the other in a 14-month-old male of experiment 3a. A most unusual tumor was a differentiated ganglioneuroma of the adrenal in a 17-month-old rat of experiment 3b.

Subcutaneous sarcomas developed at the site of injection in 15 rats, 5 in each of the last 3 experiments. Five females over 12 months of age had mammary fibroadenomas. Pulmonary metastases from the mammory tumors and sarcomas had no predilection for areas of infarction. The deliberate subcutaneous injection of MCA was carried out at the time of infarction in other experiments not reported here. In these experiments, sarcomas at the site of injection were invariably present and no alteration other than simple infarction occurred in the lungs of rats that were killed 6 months after injection.

DISCUSSION

The intravenous injection of particles of carcinogen is a method seldom used to induce tumors, perhaps because acute mortality from embolism is always high. Infarcting the lung has been a procedure rarely used

also, since this necessitates occluding only vessels of capillary dimension or grossly altering the hemodynamics of the lung. However, the method used here is not entirely new. Nearly 40 years ago, Fischer caused infarcts of the lung in rabbits by intravenously injecting olive oil combined with camphor (*34*). He considered in detail the relation of regenerative changes at the margins of healing infarcts to cancer and attempted to induce cancer by incorporating Scharlachöl, mineral oil, and tubercle bacilli in his inoculum. He failed, partly because there were no potent carcinogens available to him. The successful induction of carcinomas by this method is of importance for several reasons: The carcinomas simulate the bronchogenic carcinoma of man in cell structure and behavior, and the tumors are a good prototype for the study of problems concerning the morphology and histogenesis of lung cancer of this type. An old lesson, that lack of differentiation is neither a requisite nor a hallmark of malignant behavior, is emphasized by the contrast between the benign, undifferentiated epithelium of the bronchiectatic lesions commonly observed in rats and the high degree of squamous differentiation in many cancers and metaplastic lesions that preceded them. Furthermore, like bronchogenic carcinoma in man, the carcinomas in these rats show that remarkable modulations in cell structure can occur in a single cancer. This observation is pertinent to the problem of the relation of cell type to the prognosis and etiology of carcinoma of the lung. The method is also important because it is simple, and the incidence of carcinomas is remarkably high. We can use the method to study other potential carcinogens by adding them to the fluorocarbon and depositing them securely in the lung concentrated in areas of pulmonary damage and regeneration.

An understanding of the evolution of the carcinomas is not required for such a study. However, the mechansims involved in the genesis and progression of the tumors are important to considerations of the pathogenesis of lung cancer in general. The phenomenon of infarction in relation to the carcinomas supports a long-standing concept that pulmonary damage, fibrosis, and regeneration may be contributing factors in this disease (*34*, *35*). The microcysts of metaplastic squamous epithelium in the infarcts and the epidermoid cysts resulting from emboli of MCA crystals are responses to MCA that are similar. Both responses follow the sequestration of MCA in vascular spaces, but ischemia and infarction occur when fluorocarbon is the vehicle, while simple emboli result in minor damage and complete recovery without scarring. Is the malignant behavior of the squamous microcysts in the infarcts the result of the particular method used to deliver the carcinogen to the lung or is the process of infarction alone responsible for the malignant behavior of the tumors? Neither the experiments nor the observers are sufficiently profound to justify comment on all the problems related to the effects of solvents or the concept of tumor promotion in this system. There is little doubt that the fluorocarbon can act as a promoting agent along the lines proposed by Berenblum (*36*) and others. Fluorinated hydrocarbons

have not been tested for promoting activity, but other aliphatic hydrocarbons are known to accelerate the time of appearance of MCA-initiated tumors of the skin (*37*).

The size of the dose or the physicochemical state of the carcinogen when mixed with fluorocarbon may influence the behavior of the tumors. The highly invasive anaplastic "carcinosarcomas" of experiment 4 in which MCA was heated until completely in solution indicate that the type of tumor as well as its behavior may be influenced by such factors. The malignant lesions may be the result of an increase in the concentration of MCA in the scars, but it is difficult to imagine a concentration of MCA higher in these areas than that in the vicinity of the emboli of MCA that cause epidermoid cysts. The solubility of MCA differs in tricaprylin and fluorocarbon, but in both preparations (expts. 2 and 3) most of the MCA is present as a suspension of crystals and *more* is in solution in the MCA-tricaprylin mixture that results only in epidermoid cysts than in the MCA-fluorocarbon mixtures that results in both carcinomas and cysts. The fluorocarbon may change the chemical characteristics of the carcinogen, but a chemical reaction between the compounds is unlikely from a chemical standpoint and is not evident during preparation.

It is probable that progression to malignancy in this system is not a manifestation of difference in dose, concentration, or chemical alteration of the carcinogen. The oncogenic response to MCA may be altered simply as the consequence of tissue response to pulmonary infarction. The regenerating epithelial cells or the newly formed connective tissue of the healing infarct may contribute directly to the growth and behavior of the tumors; both have been implicated in the pathogenesis of neoplasia (*38–42*). Of particular note are the observations of Orr (*41*) and Billingham *et al.* (*42*) concerning the importance of ischemia and alterations in the connective-tissue stroma to the genesis of MCA-initiated tumors of the epidermis. The evidence for this concept in relation to the lung tumors is limited. However, it is significant that all the carcinomas probably arise in the scars of infarction, and that in lungs exposed to simple emboli of MCA, carcinomas seem to develop only at sites where the lung is subsequently damaged.

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All sections are stained with hematoxylin and eosin unless otherwise stated. Histological preparations by Mr. J. Albrecht and associates, photomicrographs by Mr. G. Gsell.

PLATE 43

FIGURE 1.—Border between viable lung tissue (*top*) and necrotic lung tissue (*bottom*) of an 8-day-old pulmonary infarct in rat receiving injections of fluorocarbon. *Note* extent of hyperplasia and proliferation of epithelium in alveoli at margin of area of ischemic necrosis. × 60

FIGURE 2.—Healing pulmonary infarct in rat receiving injections of fluorocarbon 30 days previously. A few bronchioles and many microcysts lined with cuboidal and columnar epithelium are trapped in scar tissue. × 75

FIGURE 3.—Entire scar of pulmonary infarct in rat receiving injections of fluorocarbon 12 months previously. *Note* microcysts lined with flattened epithelium and lack of squamous metaplasia. Fixation of bronchioles in scars is a common finding. × 75

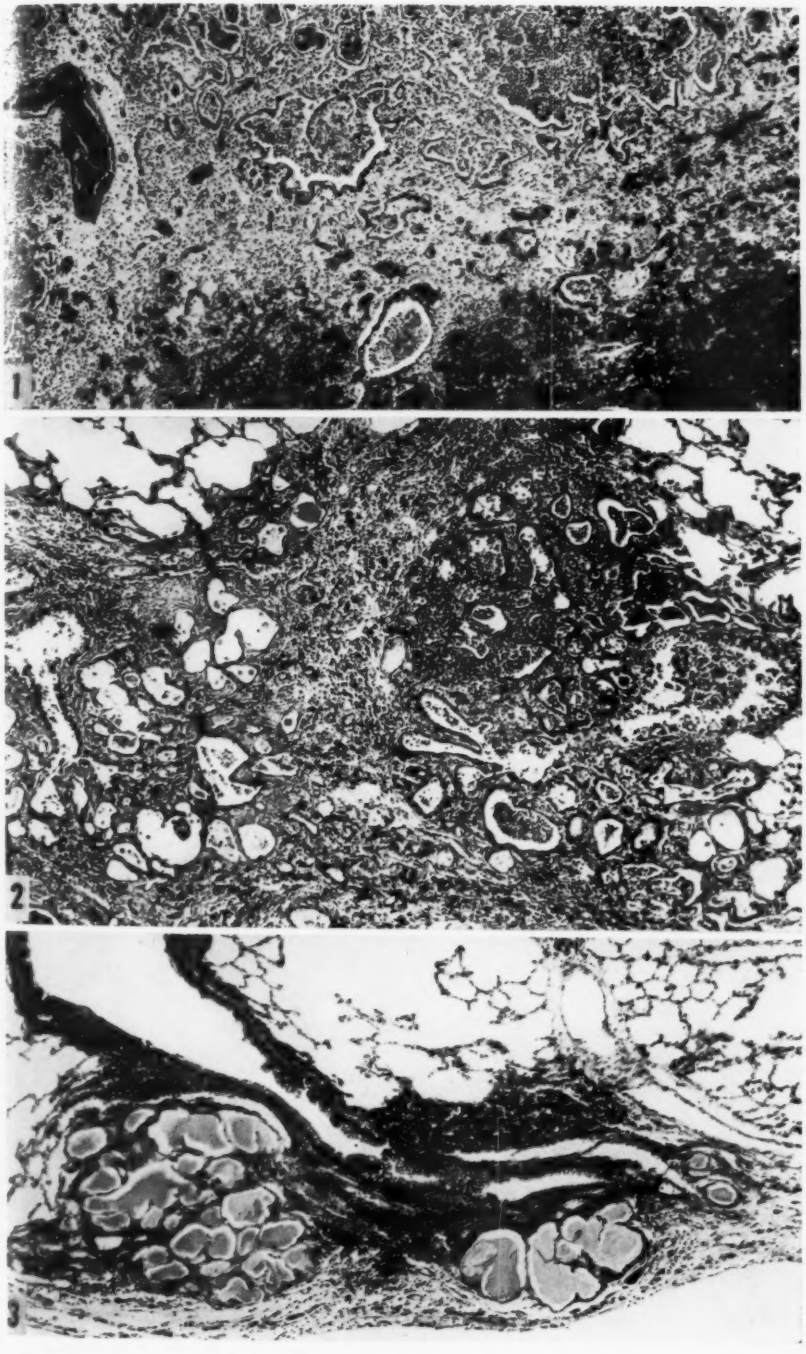
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PLATE 44

FIGURE 4.—Detail of regenerative activity of pulmonary epithelium at margin of 6-day-old infarct in rat receiving injection of methylcholanthrene mixed with fluorocarbon. During this acute stage of healing, regenerative activity is no greater than in rats receiving fluorocarbon alone. × 200

FIGURE 5.—Early squamous metaplasia in microscopic cysts of infarct scars in rat receiving injection of methylcholanthrene mixed with fluorocarbon 96 days previously. *Note* that some epithelial microcysts contain mucin-secreting cells. × 235

FIGURE 6.—Squamous metaplasia and keratinization within scar of infarct induced 4 months previously with mixture of methylcholanthrene and fluorocarbon. *Note* that metaplastic areas seem particularly localized to dense scar tissue. × 135

FIGURE 7.—Squamous metaplasia in scar of infarct induced 10 months previously with mixture of heated methylcholanthrene and fluorocarbon. Epithelial pleomorphism is more pronounced in this lesion than in most of localized microscopic foci. × 85

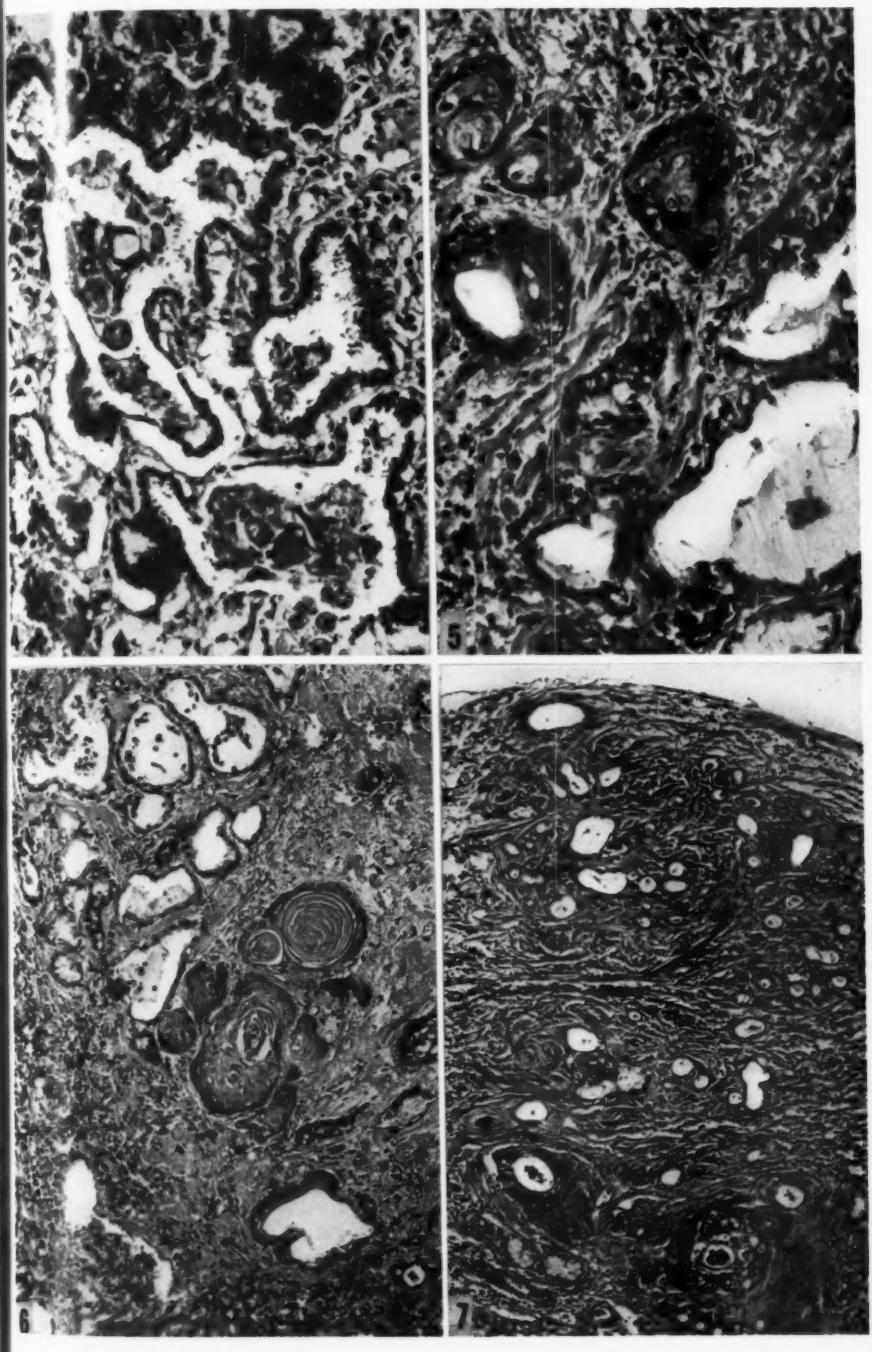

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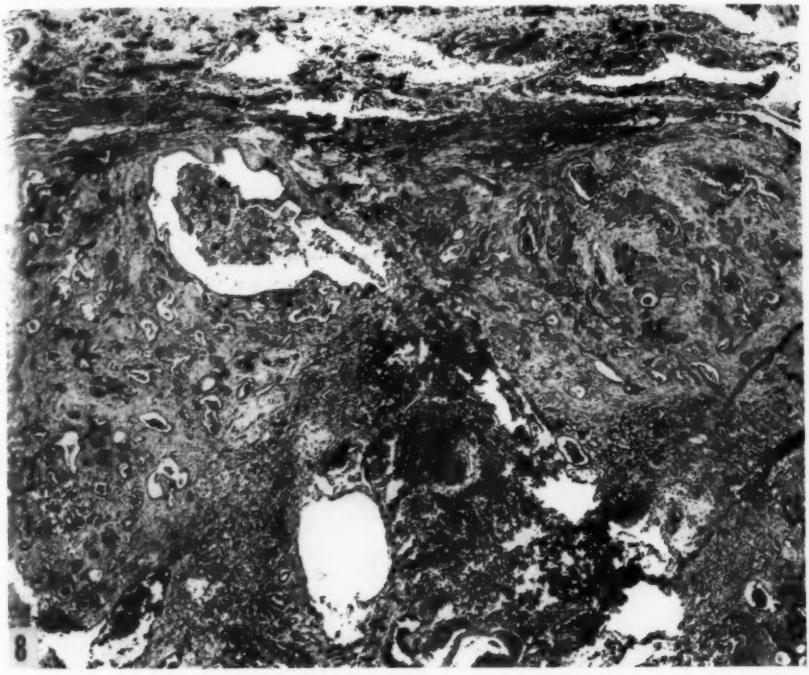
PLATE 45

FIGURE 8.—Infarct at periphery of lung with squamous epithelial lesions that are transitional between metaplastic foci illustrated in figures 6 and 7 and carcinomas illustrated in figures 10 and 11. Rat from experiment 2b received injection of methylcholanthrene and fluorocarbon 8 months before death. × 31

FIGURE 9.—Transitional lesion presumably superimposed on a site of infarction. Lesion is composed of distorted squamous epithelial elements surrounded by abundant proliferating fibroblasts. Rat from experiment 4b received injection of heated methylcholanthrene and fluorocarbon 9 months before death. × 28



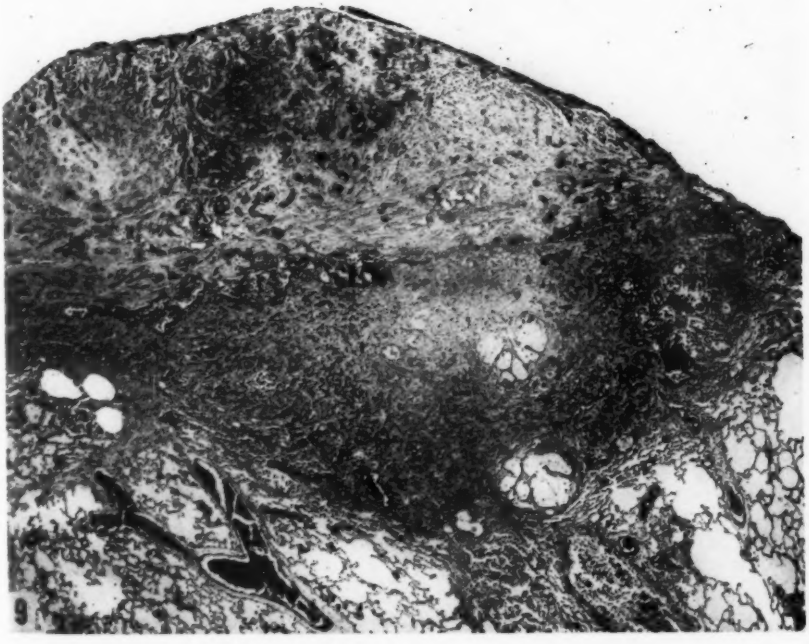
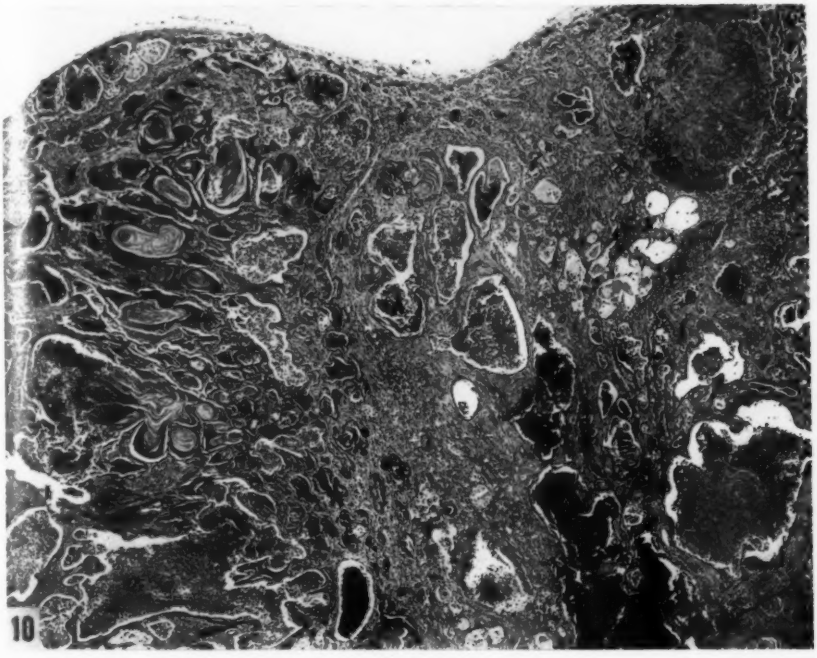


PLATE 46

FIGURE 10.—Apparent origin of metastasizing carcinoma in scar of healed infarct. *Note* cystlike keratinized areas in much of tumor. This degree of differentiation is present also in the metastases. Rat from experiment 2b received injection of methylcholanthrene in tricaprylin and fluorocarbon 8 months before death. × 24

FIGURE 11.—Apparent origin of one of the 2 carcinomas in experiment 3b. *Note* multiple, keratinized, cystic lesions throughout lower half of this lung. Rat received injection of methylcholanthrene and tricaprylin; fluorocarbon injected 4 weeks later. × 8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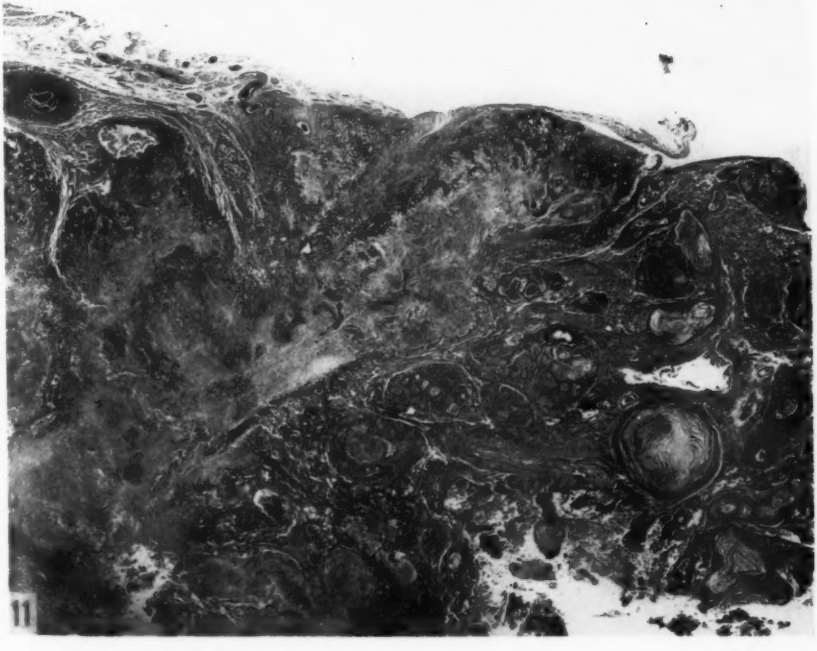
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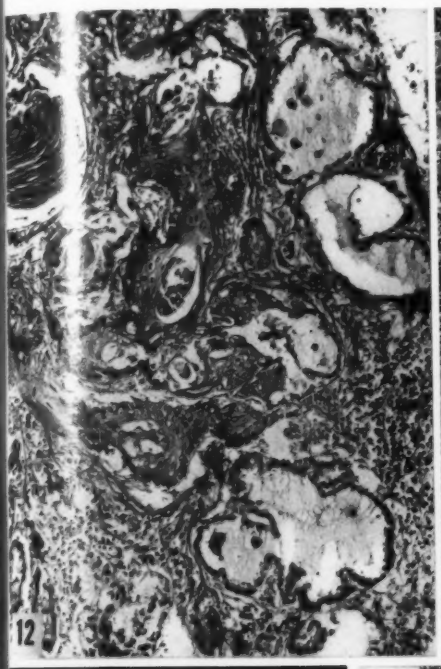
PLATE 47

FIGURE 12.—Margin of metastasizing carcinoma in peripheral area of infarction (pleural surface on *right*). Lesions of this type point to keratinized cysts within infarct scars as probable origin of most carcinomas. Rat from experiment 2a, 6 months after injection. × 125

FIGURE 13.—Metastasizing epidermoid carcinoma in rat receiving injections of mixture of methylcholanthrene, tricaprylin, and fluorocarbon 13 months before death. *Note* evidence of probable origin of this tumor from bronchial epithelium at margin of peripheral area of infarction. × 100

FIGURE 14.—Gross specimen of *left half* of thoracic cavity. Left lung has been removed, dissected, and replaced. *Note* extensive involvement of *lower half* of lung, thorax, and diaphragm. Exact origin of this carcinoma could not be detected. Rat from experiment 2a, 11 months after injection. × 1

FIGURE 15.—Extensive involvement of *lower half* of left lung with invasion of diaphragm by epidermoid carcinoma (exact site of origin undetermined). Rat from experiment 4b, 8 months after injection. × 6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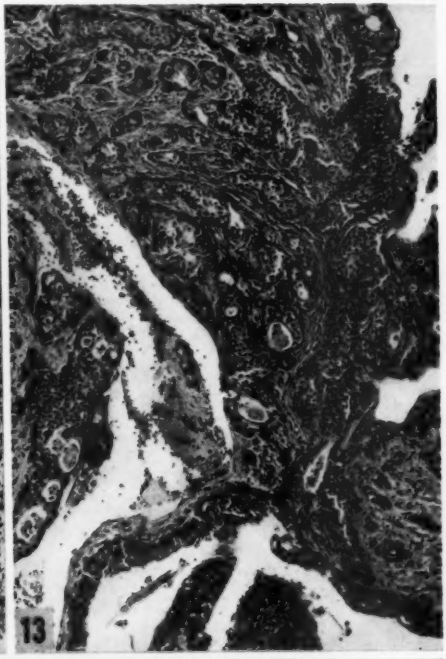
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PLATE 48

FIGURE 16.—Cell structure typical of most of carcinomas in experiments 1 and 2. *Note* tendency to differentiate as squamous epithelium. Rat from experiment 1 received injection 12 months before death. × 135

FIGURES 17, 18, and 19.—Various areas in single carcinoma of lung from rat in experiment 2b killed 10 months after receiving injection. *Note* apparent modulations in cell structure from keratinizing squamous cells in figure 17 to mucin-secreting cells in figures 18 and 19. × 210

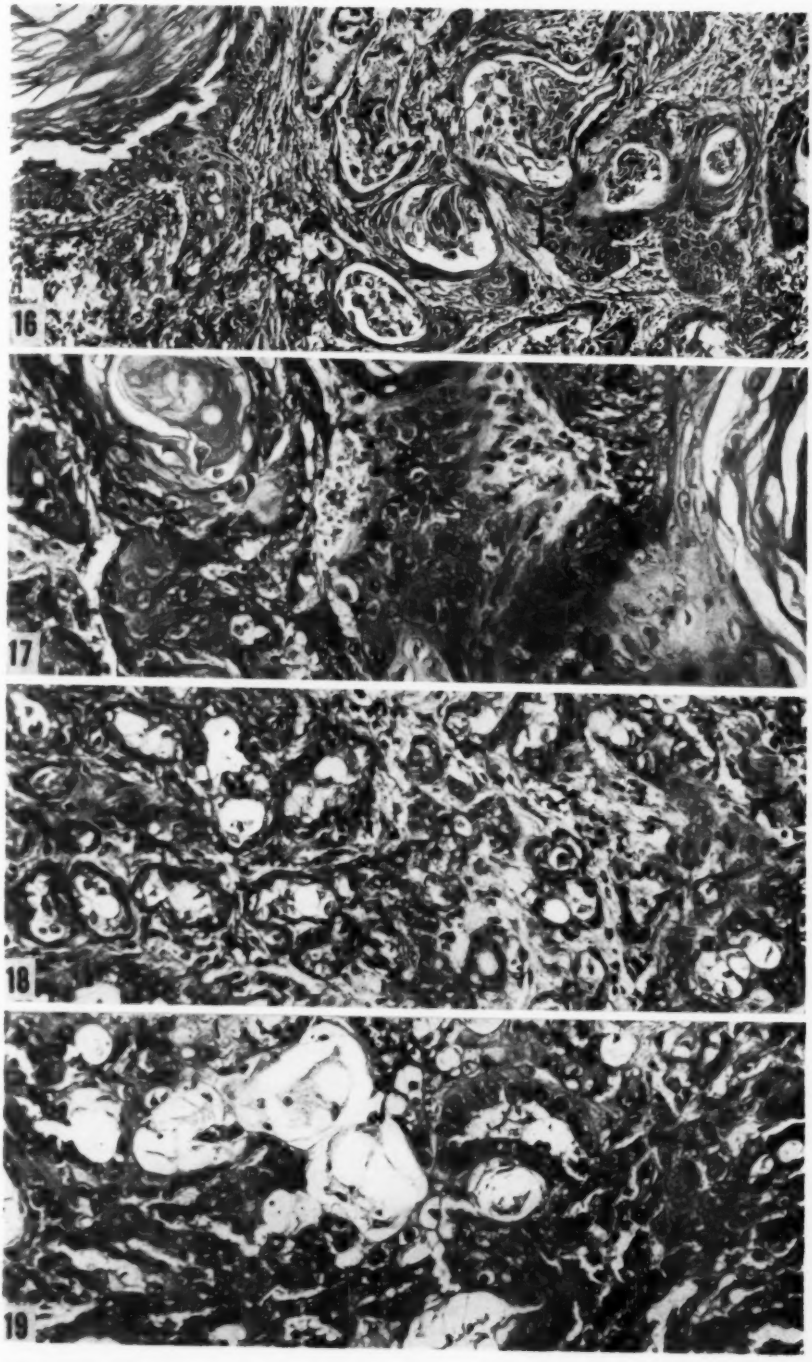
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PLATE 49

FIGURE 20.—Metastasis to tracheobronchial lymph node at hilus of lung. Primary carcinoma localized in caudal half of *left* lung. *Note* well-differentiated keratinized epithelium in some areas. Rat from experiment 2b, 12 months after injection. × 135

FIGURE 21.—Metastatic embolic glomerulonephrosis, a rare metastatic lesion in experimental animals. Primary epidermoid carcinoma of *left* lung in rat from experiment 2a, 10 months after injection. × 235

FIGURE 22.—Epidermoid carcinoma of *left* lung invading vertebrae. *Note* differentiated squamous cells of tumor and marked osteogenic reaction of vertebral bone on *upper right*. Primary epidermoid carcinoma of *left* lung in rat from experiment 2a, 10 months after injection. × 84

FIGURE 23.—Different area of same invasive carcinoma illustrated in figure 22 shows pseudoglandular structure of carcinoma near invaded bone. × 235

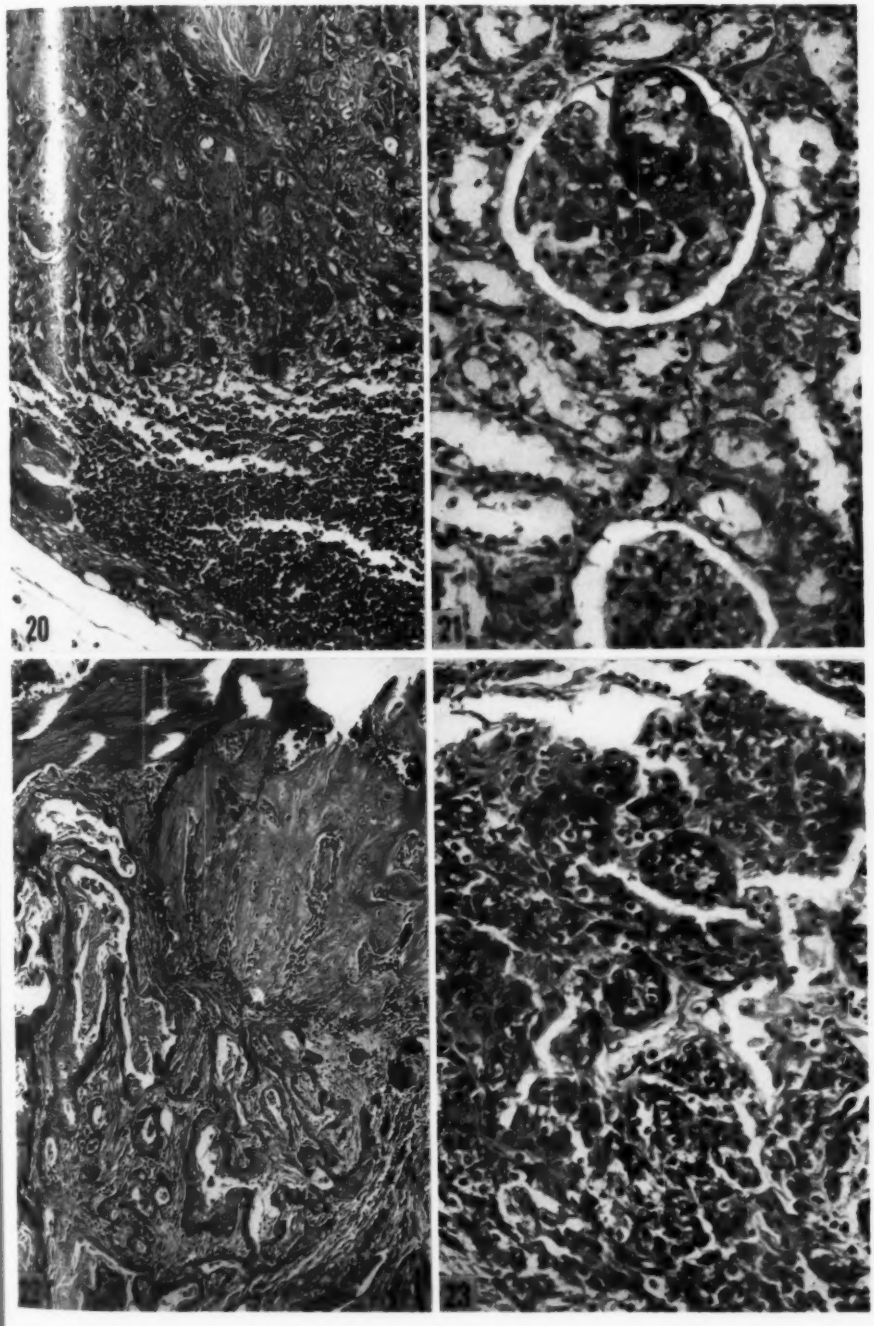
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PLATE 50

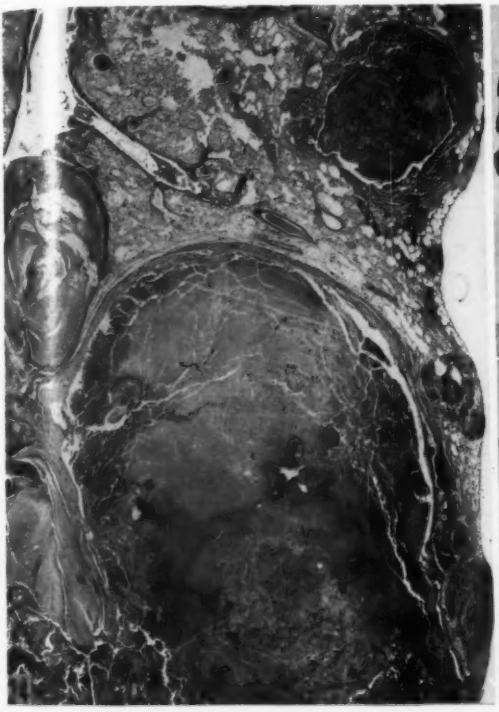
FIGURE 24.—Multiple epidermoid cysts of lung in rat from experiment 3b, 9 months after receiving injection of methylcholanthrene in tricaprylin. × 6

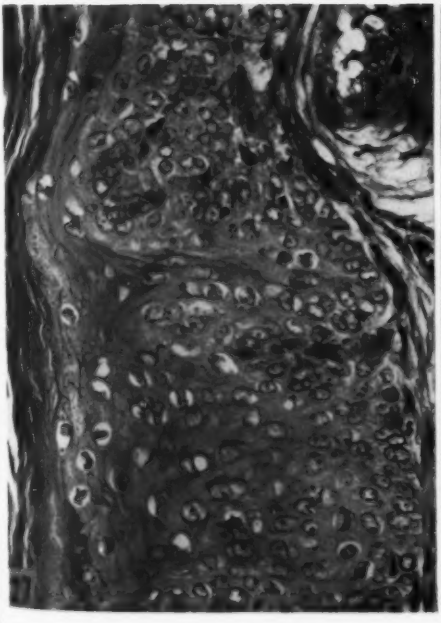
FIGURE 25.—Gross appearance of larger epidermoid cysts in rat from experiment 3a, 10 months after receiving injection of methylcholanthrene in tricaprylin. *Compare* with figure 26. × 1

FIGURE 26.—Gross appearance of advanced bronchiectasis in rat receiving injection of fluorocarbon 16 months before death. *Note* infarct scars in *middle right* lobe. × 1

FIGURE 27.—Detail of wall of epidermoid cyst. *Note* stratification, keratinization, and apparent stratum granulosum. *Compare* with figure 28. × 290

FIGURE 28.—Detail of wall of a bronchiectatic cavity. *Note* lack of keratinized epithelium and disorganized metaplasia. For further illustrations of this lesion *see* (*33*). Eighteen-month-old rat from experiment 3a. × 235





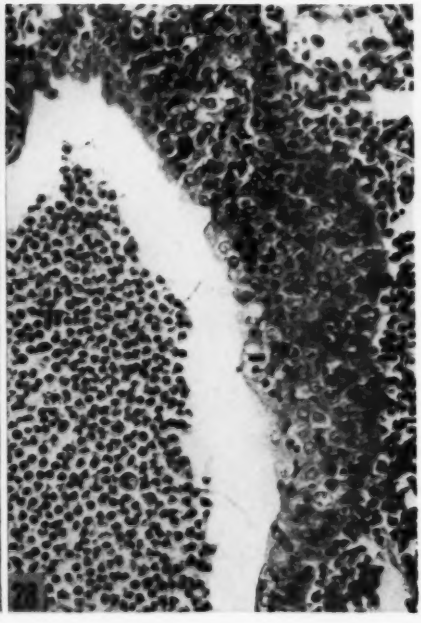
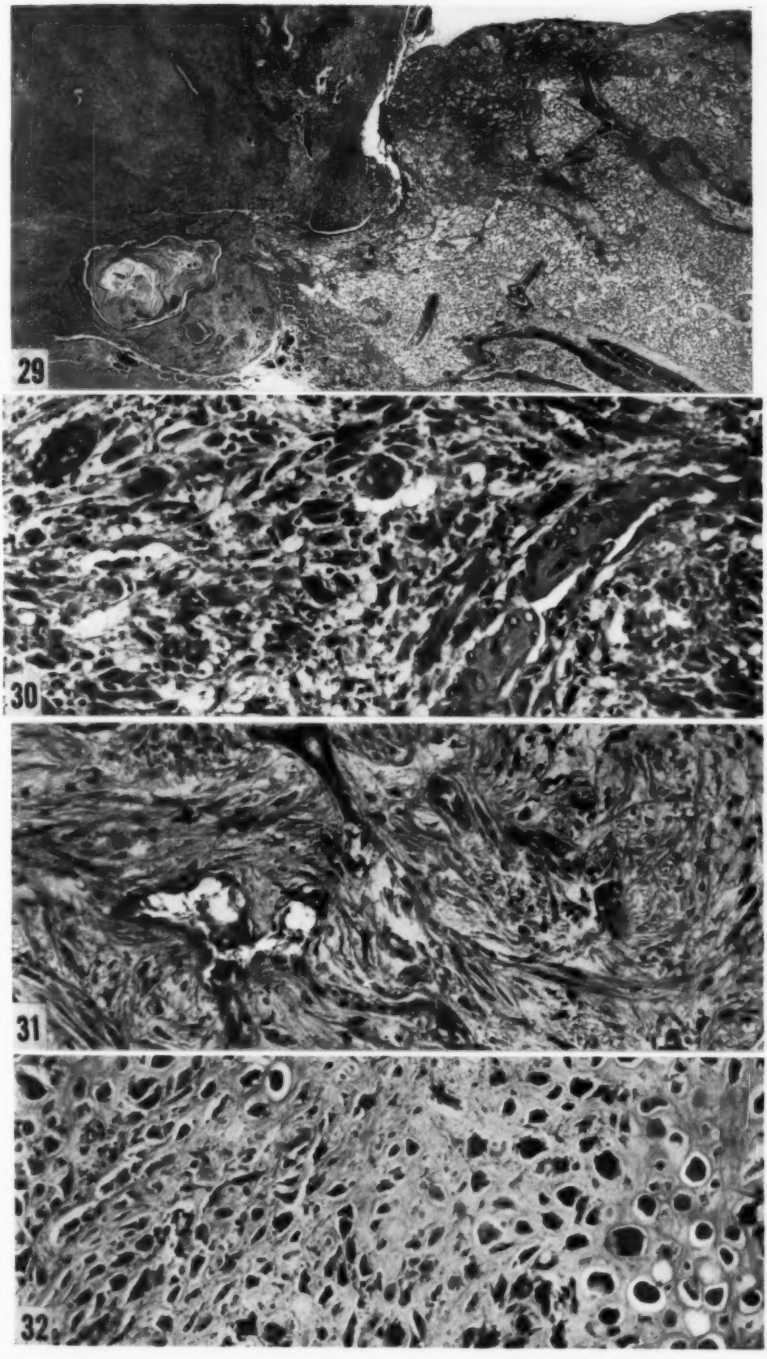


PLATE 51

FIGURE 29.—Low-power view of highly malignant tumor (carcinosarcoma?) of lung, 7 months after rat received injection of heated methylcholanthrene and fluorocarbon. *Note* keratinized cystic area in scarred portion of periphery of lung at *lower left*. × 10

FIGURES 30, 31, and 32.—Different microscopic fields from same tumor illustrated in figure 29. Figure 30: cells of apparent epithelial origin merge with pleomorphic spindle and giant cells. Figure 31: tubular pattern of tumor epithelium in desmoplastic stroma. Figure 32: anaplastic tumor cells in dense nonfibrous matrix. × 200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Tolerance Induced by Parity in Mice Incompatible at the *H-2* Locus[1, 2]

EDWARD J. BREYERE[3] *and* MORRIS K. BARRETT,
Laboratory of Biology, National Cancer Institute,[4]
Bethesda, Maryland

SUMMARY

BALB/c (*H-2*^{d}) female mice were divided into 3 groups and mated to males of the following strains to produce from 1 to 8 litters: C3H (*H-2*^{k}), DBA/2 (*H-2*^{d}), and BALB/c. A fourth group was retained as virgins. After the parous females had their last litter, all 4 groups were challenged with plasma-cell tumor SS (70429), of C3H origin. The tumor grew in 59 percent of females parous by C3H males, in less than 1 percent of those parous by BALB/c males, and in none of the others. These findings are presented as evidence that tolerance in an apparently *H-2* incompatible system can be induced by strain-specific matings. As in previous studies, tolerance appeared to increase with multiparity. Approximately half the tumors in specifically parous females grew in the form of a ring, a phenomenon apparently peculiar to this situation.—J. Nat. Cancer Inst. 27: 409–417, 1961.

WE PREVIOUSLY reported that immunological tolerance to homologous grafts of tumor or skin was seen in postpartum female mice parous by males of the strain from which the grafts originated. Tolerance increased with multiparity and was effective both in natural and induced resistance. Furthermore, this effect was long lasting and specific matings would partially abrogate a previously induced resistance (*1*, *2*). Prehn has reported that females parous by intrastrain matings are tolerant of male skin isografts, and that tolerance increased with multiparity (*3*). His results and ours were, however, obtained with materials isogenic with respect to the *H-2* histocompatibility locus. Using a donor-host combination that differed at this locus, Medawar and Sparrow did not detect tolerance induced by parity (*4*). It seemed possible that their negative findings may have resulted from the fact that tolerance to antigens governed by this locus may not be induced by strain-specific matings. Genetic dif-

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[2] This investigation was supported in part by fellowship CF-5857 from the National Cancer Institute, National Institutes of Health, Public Health Service.
[3] *Present address:* Department of Biology, American University, Nebraska and Massachusetts Aves., Washington 16, D.C.
[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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ferences involving *H-2* alleles give rise to a high degree of transplantation immunity and might "mask" tolerance to weaker transplantation antigens.

The following experiments were performed in an attempt to test this possibility. Evidence is presented indicating that in this particular donor-host combination, tolerance to *H-2* antigens results from specific parity.

MATERIALS AND METHODS

All host animals were BALB/cAnN ($H\text{-}2^d$)[5] female mice. They were divided into 4 groups and mated as follows: the first to C3H/HeN ($H\text{-}2^k$) males to produce from 1 to 8 litters; a second similarly mated to BALB/c males; a third to DBA/2JN ($H\text{-}2^d$) males to produce from 5 to 6 litters, and the fourth retained as virgins. Ages were matched as nearly as possible, and parous females were inoculated with the tumor on an average of 15 days after their last litter.

The tumor used for challenge was an undifferentiated plasma-cell neoplasm of C3H origin designated as "SS." It is a subline of 70429 (*5*) and had been maintained in this laboratory for over 150 transplant generations prior to these procedures. At the present time it grows progressively in all C3H/HeN mice, but is not homotransplantable to strain BALB/c. During the course of these and other concurrent experiments, it grew in only 1 of 362 BALB/c mice tested. Each recipient received a single subcutaneous inoculation of 0.025 cc of a 50 percent tumor suspension in saline in the right flank. With the exception of tumor-bearing animals that were killed for histological sections, the word "take" refers to the death of a host bearing a progressively growing tumor.

RESULTS

The tumor took in 59 percent (89/152) of females parous by C3H males, not in virgins (0/53), and in only 1 of the females parous by intrastrain matings (1/169). This one positive observation in the last group was probably the result of host variation rather than a change in the tumor, since this tumor failed to take on subsequent transfer in strain BALB/c. The percentage of takes seemed to increase with multiparity by C3H males, but the percentages were irregular and only followed a general pattern (table 1). A comparison between individual groups was therefore difficult to draw, but the effect of increased multiparity is illustrated by the fact that the tumor grew in 33 percent (18/54) of females with 1 through 3 litters and increased to an average of 72 percent (71/98) in females that had 4 through 8 litters ($P<0.001$). The tumor did not take in the small group of females that had 5 or 6 litters by DBA/2 males (0/10). These are not shown in the table.

[5]We are indebted to Dr. G. Hoecker for testing our sublines with his *H-2* specific antiserums.

TABLE 1.—Growth of C3H plasma-cell tumor SS (70429) in BALB/c females parous by intrastrain and interstrain matings

Number of litters	Females previously mated to:*			
	C3H males		BALB/c males	
	Takes/total	Percent	Takes/total	Percent
1	4/16	25	0/18	0
2	9/20	45	0/17	0
3	5/18	28	0/29	0
4	15/23	65	1/33	3
5	18/23	78	0/32	0
6	12/17	71	0/22	0
7	17/21	81	0/14	0
8	9/14	64	0/4	0
Total	89/152	59	1/169	<1

*The tumor did not take in virgin females 0/53.

An unexpected finding was an unusual growth pattern of the tumor in 48 percent of the tumor-bearing, specifically parous BALB/c females. During the first week, in all mice including those of the strain of origin (C3H), the tumor grew at the site of inoculation in the form of a rounded swelling (fig. 1). In the majority (52 percent) of tumor-bearing BALB/c females and in mice of the strain of origin, this mass merely increased in size, in the manner usual for most tumors, until death of the host occurred. In the other tumor-bearing BALB/c females, this period of local growth was followed by one in which the tumor grew in an annular form or "doughnut." The central portion of the tumor first became flattened (fig. 2), then umbilicate at about 2 weeks (fig. 3), and finally a ring of tumor was formed by the 3d week (fig. 4). When the inoculation site was previously tattooed with India ink or when blood charcoal was included in the tumor inoculum, these identifying marks remained in the center of the ring, which gave the impression that the tumor had migrated away from the site of inoculation. The annular tumors increased in diameter by an apparent growth at the periphery with simultaneous regression at the inner border, until their hosts died during the 3d or 4th week.

Grossly, the onset of ring formation was marked by a return of the skin to a normal texture in the center. Histological sections of this area in a fully developed ring showed an almost normal architecture of the skin and its adjacent connective tissues (fig. 5). There was a small amount of granulation tissue and edema with a slight increase in fibrous elements. The panniculus and epidermis were normal. In contrast to the center, the annular tumor itself was composed of a high concentration of tumor cells that had invaded the dermis, panniculus, and loose connective tissues. Occasional breaks in the skin were found over the ring that were probably the result of mechanical abrasion; otherwise necrosis was absent.[6]

[6] The authors are grateful to Dr. Thelma Dunn of the Laboratory of Pathology, National Cancer Institute, for her review of microscopic slides.

DISCUSSION

These results confirm our previous observations and also indicate that an apparent incompatibility at the *H-2* locus can be overcome by the induction of tolerance by specific parity. This tumor has not been specifically tested for susceptibility to *H-2* differences except that it does not grow in normal DBA/2 or BALB/c hosts. In previous studies involving an *H-2* compatible tumor-host combination, maternal tolerance was best demonstrated by prior immunization with homologous blood (*1*) or skin (*2*), but this was unnecessary in the present instance as the tumor was not homotransplantable in control animals. Similarly, Snell has reported that prior immunization was unnecessary for the development of coisogenic strains with an *H-2* incompatibility, as this locus produces a sufficient barrier to homologous grafts (*6*). Previous studies involving the transplantation of this C3H tumor into normal resistant backcross or F_2 hosts have indicated that this tumor differs from the BALB/c strain by at least 2 or 3 factors (*7* and unpublished data). The negative findings of Medawar and Sparrow may be the result of their particular test system and not from an *H-2* difference *per se* (*4*).

In this study it was apparent that the tolerance was incomplete with respect to the total number of animals tested, since the tumor did not take in 100 percent of any group of females. A similar result was previously obtained with skin homografts, as none of these survived permanently (*2*). The ring formation observed with this tumor in the homologous strain is interesting. When the tumor was grown in the strain of origin, this pattern was not observed in the course of many transplant generations in this laboratory, nor was it observed by Dunn in her line of this tumor (personal communication). It is tempting to speculate that ring formation resulted from a gradually developing local immunity, since the tumor appeared to regress or migrate away from the inoculation site, but at present no information is available as to the responsible mechanism, and further studies are required before any definite statements can be made.

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PLATES

PLATE 52

Photographs of different BALB/c females that show representative stages in development of annular tumors. Hair was removed from tumor areas with a depilatory cream.

FIGURE 1.—Tumor growth at site of inoculation at 11 days. Inoculation site is marked with India ink.

FIGURE 2.—Beginning of annular tumor formation with central flattening.

FIGURE 3.—Tumor showing umbilication.

FIGURE 4.—Fully developed annular tumor at 26 days. Transverse section of this tumor is shown in figure 5. *Note* absence of central necrosis. Ventrally, tumor has not invaded the dermis.

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PLATE 53

FIGURE 5.—Dorsoventral transverse section of tumor seen in figure 4; photograph taken on day of the gross photograph (day 26). Site of inoculation is marked by blood charcoal. Central portion shows absence of tumor cells, slight amount of edema, and granulation tissue. Van Gieson's stain. × 8

5

BREYERE AND BARRETT

Smoking Habits of Men and Women [1]

E. CUYLER HAMMOND, *Sc.D.*, *and* LAWRENCE GARFINKEL, *M.A.*, *Statistical Research Section, American Cancer Society, Inc., New York, New York*

SUMMARY

Between October, 1959, and February, 1960, approximately 1,085,000 people in 25 States answered questionnaires which included questions on smoking habits. The present study is based on a sample of 43,068 of these subjects. Some of the findings were as follows: Exposure to cigarette smoke is far less in the female than in the male population, as indicated by percent of heavy cigarette smokers, degree of inhalation, nicotine and tar content of cigarettes, and age at which smoking was begun. Cigar and pipe smokers tend to inhale the smoke to a far lesser degree than do cigarette smokers. A larger proportion of college graduates than those with less education have given up cigarette smoking, and a larger proportion smoke low-tar, low-nicotine cigarettes. The proportion of cigarette smokers is less in farmers, teachers, members of the legal profession, clergymen, doctors, dentists, and veterinarians than in the general population. The proportion of ex-cigarette smokers and of men smoking low-tar, low-nicotine cigarettes is higher in these occupational groups (except farmers) than in the general population.—J. Nat. Cancer Inst. 27: 419–442, 1961.

HAENSZEL, SHIMKIN, and MILLER (*1*) have reported on the smoking habits in a large random sample of the adult population of the United States as of February, 1955, which provides valuable information on smoking in relation to such variables as age, sex, occupation, and urban versus rural residence. This has been useful in studies of these factors in relation to death rates from lung cancer and other diseases. The purpose of the present paper is to provide more recent data plus information on several additional variables such as inhalation, nicotine and tar content of cigarette smoke, and use of chewing tobacco and snuff, all of which may be important in relation to health.

MATERIALS AND METHODS

Between October 1, 1959, and February 15, 1960, volunteer researchers of the American Cancer Society, Inc., enrolled approximately 1,085,000 [2]

[1] Received for publication March 27, 1961.

[2] In addition, the Seventh Day Adventist Church enrolled 37,000 of their members for us, so the total study includes about 1,122,000 subjects. However, the Seventh Day Adventist group is excluded from the present report since they are not typical of the general public in respect to smoking habits.

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people in a prospective epidemiological study planned for a 6-year period of follow-up (*2*). The subjects were enrolled in family groups, *i.e.*, households, in each of which there was at least one person over the age of 45. Every person in these families over 30 years of age was requested to fill out a detailed questionnaire on family history, diseases, physical complaints, environmental exposures, habits, occupation, residence, etc. Questions on smoking habits and the use of chewing tobacco and snuff are shown in text-figures 1 and 2 for males and females, respectively. The study area covered 1,121 counties in 25 States.[3] Cities and towns of all sizes and types were included as well as rural areas. Insofar as possible,

3. Do you now smoke? Yes ☐ No ☐
   If "yes,": a) How many cigarettes do you usually smoke a day?_____
   b) How many cigars do you usually smoke a day?_____
   c) How many pipefuls of tobacco do you usually smoke a day?_____
4. If you now smoke cigarettes:
   a) About how much do you inhale when smoking cigarettes?
   Do not inhale ☐ Inhale Slightly ☐ Inhale Moderately ☐ Inhale Deeply ☐
   b) What type do you smoke? Filter-tip ☐ Without filter-tip ☐
   c) What brand do you usually smoke?_____
   d) How old were you when you started smoking cigarettes?_____
5. If you now smoke cigars, about how much do you inhale when smoking cigars?
   Do Not Inhale ☐ Inhale Slightly ☐ Inhale Moderately ☐ Inhale Deeply ☐
6. If you now smoke a pipe, about how much do you inhale when smoking a pipe?
   Do Not Inhale ☐ Inhale Slightly ☐ Inhale Moderately ☐ Inhale Deeply ☐
7. If you do not smoke cigarettes now, did you ever smoke cigarettes regularly? Yes ☐ No ☐
   If "yes,": a) How long has it been since you last smoked cigarettes regularly?_____
   b) How many cigarettes did you usually smoke per day?_____
   c) Why did you stop smoking cigarettes?_____
8. If you do not smoke cigars now, did you ever smoke cigars regularly? Yes ☐ No ☐
9. If you do not smoke a pipe now, did you ever smoke a pipe regularly? Yes ☐ No ☐
10. Do you chew tobacco or use snuff? Never ☐ Occasionally ☐ Regularly ☐

TEXT-FIGURE 1.—Questions on smoking for men.

3. Do you now smoke? Yes ☐ No ☐
   If "yes,": a) How many cigarettes do you usually smoke a day? _____
   b) About how much do you inhale when smoking cigarettes?
   Do not inhale ☐ Inhale slightly ☐ Inhale moderately ☐ Inhale deeply ☐
   c) What type do you smoke? Filter-tip ☐ Without filter-tip ☐
   d) What brand do you usually smoke? _____
   e) How old were you when you started smoking cigarettes? _____
4. If you do not smoke cigarettes now, did you ever smoke cigarettes regularly? Yes ☐ No ☐
   If "yes," a) How long has it been since you last smoked cigarettes regularly? _____
   b) How many cigarettes did you usually smoke per day? _____
   c) Why did you stop smoking cigarettes? _____

TEXT-FIGURE 2.—Questions on smoking for women.

[3] Arizona, California, Florida, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Maryland, Massachusetts, Michigan, Minnesota, Mississippi, Missouri, New York, North Carolina, Ohio, Oregon, Pennsylvania, South Carolina, Tennessee, Texas, and Virginia.

all segments of the population were represented in the sample except for illiterates, migrants who could not have been traced, and persons too ill to fill out a questionnaire. The distribution of the study population by age, sex, and education has been presented elsewhere (*3*).

Follow-up information will not be available for some time; and, because of the large number of lengthy questionnaires, it will take several years to code all the original data. For this reason, we drew a sample of 44,848 questionnaires and coded certain items which are of interest even without follow-up data. The sample was selected in such a way that all study areas are proportionally represented.

Of the 44,848 questionnaires, 1,495 were seriously incomplete, *e.g.*, age not stated, one or more pages skipped, or inadequate answers to questions on smoking, and 285 were filled out by persons under 30 years of age. The present analysis is based upon the remaining 43,068 questionnaires (18,697 from men and 24,371 from women) which were satisfactory for our purpose.

GENERAL FINDINGS ON SMOKING

Table 1 gives a general summary of smoking habits by sex and 10-year age groups. In various studies on smoking habits the questions have been asked in somewhat different ways. Therefore, the figures from different studies may not be strictly comparable. In this study there was no question specifically asked about "occasional smoking." As a result, very few people identified themselves as "occasional smokers." Presumably, most people who rarely smoked recorded themselves as nonsmokers while people who smoked nearly every day were generally classified as regular smokers. This is what we desired for later analysis after the follow-up is complete, but it must be kept in mind if one compares these findings with those in some other studies.

Within the age range studied, the proportion of current regular smokers decreased with age and was considerably less among females than among males. For example, in age group 30 to 39, 66.8 percent of the men and 41.7 percent of the women were current regular smokers, while in age group 80 and older, 32.5 percent of the men and only 3.0 percent of the women were current regular smokers.

Among men, type of smoking varied with age. For example, in age group 30 to 39, 51.2 percent of the men currently smoked only cigarettes, 8.0 percent smoked cigarettes and also cigars or pipes, and 7.5 percent smoked cigars or pipes or both, but not cigarettes. In age group 80 and older, 8.1 percent of the men currently smoked only cigarettes, 2.0 percent cigarettes and also cigars or pipes, and 22.3 percent cigars or pipes or both but not cigarettes.

Chewing Tobacco and Snuff

Altogether 4.0 percent of the men said that they regularly used chewing tobacco or snuff. The percentage increased with age as follows: 0.8 per-

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TABLE 1.—Summary of smoking habits by age and sex

Smoking habit	Total		Age 30–39		Age 40–49		Age 50–59		Age 60–69		Age 70–79		Age 80+	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Males:														
Never smoked	3, 942	21. 1	234	19. 7	956	18. 2	1, 338	19. 4	884	23. 1	426	32. 8	104	42. 3
Occasional only	104	0. 6	4	0. 3	34	0. 6	35	0. 5	21	0. 5	10	0. 8	—	—
Ex-regular smokers	3, 659	19. 6	155	13. 0	859	16. 3	1, 332	19. 4	933	24. 4	321	24. 7	59	24. 0
Smoked, pattern uncertain*	54	0. 3	2	0. 2	7	0. 1	15	0. 2	18	0. 5	9	0. 7	3	1. 2
Current regular smoking:														
Total	10, 938	58. 5	794	66. 8	3, 404	64. 7	4, 159	60. 5	1, 968	51. 5	533	41. 0	80	32. 5
Cigarette only	7, 501	40. 1	609	51. 2	2, 520	47. 9	2, 970	43. 2	1, 135	29. 7	247	19. 0	20	8. 1
Cigarette and cigar	564	3. 0	41	3. 4	174	3. 3	207	3. 0	111	2. 9	27	2. 1	4	1. 6
Cigarette and pipe	527	2. 8	42	3. 5	177	3. 4	173	2. 5	115	3. 0	19	1. 5	1	0. 4
Cigarette, cigar, pipe	142	0. 8	13	1. 1	32	0. 6	63	0. 9	30	0. 8	4	0. 3	—	—
Cigar only	1, 032	5. 5	41	3. 4	244	4. 6	360	5. 2	267	7. 0	100	7. 7	20	8. 1
Pipe only	751	4. 0	27	2. 3	155	2. 9	246	3. 6	204	5. 3	99	7. 6	20	8. 1
Cigar and pipe	421	2. 3	21	1. 8	102	1. 9	140	2. 0	106	2. 8	37	2. 8	15	6. 1
Total	18, 697	100. 0	1, 189	100. 0	5, 260	100. 0	6, 879	100. 0	3, 824	100. 0	1, 299	100. 0	246	100. 0
Females:														
Never smoked	16, 170	66. 3	1, 167	50. 1	4, 260	54. 5	5, 215	67. 4	3, 653	82. 6	1, 491	89. 5	384	95. 5
Occasional only	108	0. 4	7	0. 3	46	0. 6	34	0. 4	17	0. 4	4	0. 2	—	—
Ex-regular smokers	1, 323	5. 4	170	7. 3	551	7. 1	400	5. 2	156	3. 5	41	2. 5	5	1. 2
Smoked, pattern uncertain*	126	0. 5	13	0. 6	37	0. 5	44	0. 6	24	0. 5	7	0. 4	1	0. 2
Current regular cigarette smoking	6, 644	27. 3	970	41. 7	2, 920	37. 4	2, 046	26. 4	574	13. 0	122	7. 3	12	3. 0
Total	24, 371	100. 0	2, 327	100. 0	7, 814	100. 0	7, 739	100. 0	4, 424	100. 0	1, 665	100. 0	402	100. 0

*Has smoked but uncertain as to present habits; includes a few women who smoke pipes or cigars only.

cent in age group 30 to 39; 2.0 percent in age group 40 to 49; 3.4 percent in age group 50 to 59; 6.4 percent in age group 60 to 69; and 10.0 percent in age group 70 and older. Of men who never smoked regularly, 6.9 percent regularly used chewing tobacco or snuff, this percentage increasing from 1.3 percent in age group 30 to 39 to 14.1 percent in age group 70 and older. The proportion of men who said that they regularly used chewing tobacco or snuff was: 1) 1.8 percent among current regular cigarette smokers (regardless of other smoking); 2) 6.1 percent among men who currently smoked only cigars; 3) 4.7 percent among men who currently smoked only pipes; 4) 5.5 percent among men who currently smoked cigars and pipes but not cigarettes; and 5) 5.1 percent among ex-regular smokers.

Ex-Smokers

In table 1 the category labeled "ex-regular smokers" includes all persons who once smoked regularly (cigarettes, pipes, or cigars) but who were not smoking regularly at the time they answered the questionnaire. Among males, the proportion of ex-regular smokers (expressed as a percentage of the total male population) increased with age. Among females the corresponding proportion decreased with age.

If one considers only potential ex-regular smokers, *i.e.*, all those who ever smoked regularly, the percentage of ex-regular smokers increased with age among females as well as among males. For males, these percentages are 16.3 percent in age group 30 to 39; 20.2 percent in age group 40 to 49; 24.3 percent in age group 50 to 59; 32.2 percent in age group 60 to 69; 37.6 percent in age group 70 to 79; and 42.4 percent in age group 80 and older. For females, the corresponding percentages are 14.9 percent in age group 30 to 39; 15.9 percent in age group 40 to 49; 16.4 percent in age group 50 to 59; 21.4 percent in age group 60 to 69; 25.2 percent in age group 70 to 79; and 29.4 percent in age group 80 and older.

Table 2 shows the number of subjects with a history of regular cigarette smoking (irrespective of pipe or cigar smoking) and the number and proportion of these who had stopped smoking cigarettes regularly before they filled out the questionnaire. The male ex-cigarette smokers are further subdivided by the number of years since they gave up cigarette smoking (some of them continued to smoke cigars or pipes).

The proportion of ex-cigarette smokers (in relation to the number of subjects with a history of regular cigarette smoking) increases with age and is greater among males than females. The sex difference in this respect is partly but not entirely due to the fact that: 1) Some men with mixed smoking habits gave up cigarette smoking but continued to smoke pipes or cigars. 2) Some men who once smoked only cigarettes later gave them up and started to smoke pipes or cigars. The proportion of men who once smoked cigarettes regularly but were currently smoking only pipes and cigars was: 1) 3.6 percent in age group 30 to 39; 2) 5.2 percent in age group 40 to 49, 3) 5.3 percent in age group 50 to 59; 4)

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TABLE 2.—Ex-regular cigarette smokers by years since last regular cigarette smoking

Sex and age	Number with history of regular cigarette smoking†	Ex-regular cigarette smokers*: Total ex-cigarette		Stopped <1 year		Stopped 1–2 years		Stopped 2–3 years		Stopped 3–4 years		Stopped 4–5 years		Stopped 5+ years		Years unknown	
		Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Males:																	
30–39	877	172	19.6	31	3.5	17	1.9	20	2.3	16	1.8	6	0.7	81	9.2	1	0.1
40–49	3,876	973	25.1	120	3.1	50	1.3	77	2.0	72	1.9	48	1.2	595	15.4	11	0.3
50–59	4,829	1,416	29.3	150	3.1	90	1.9	101	2.1	84	1.7	68	1.4	917	19.0	6	0.1
60–69	2,302	911	39.6	73	3.2	48	2.1	56	2.4	55	2.4	44	1.9	630	27.4	5	0.2
70+	602	280	46.5	15	2.5	12	2.0	18	3.0	16	2.7	10	1.7	205	34.1	4	0.7
Total	12,486	3,752	30.0	389	3.1	217	1.7	272	2.2	243	1.9	176	1.4	2,428	19.4	27	0.2
Females:																	
30–39	1,140	170	14.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—
40–49	3,471	551	15.9	—	—	—	—	—	—	—	—	—	—	—	—	—	—
50–59	2,446	400	16.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
60–69	730	156	21.4	—	—	—	—	—	—	—	—	—	—	—	—	—	—
70+	180	46	25.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total	7,967	1,323	16.6	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*Includes ex-regular cigarette smokers who currently smoke pipes or cigars.
†Includes all subjects with a history of regular cigarette smoking irrespective of pipe or cigar smoking.

7.9 percent in age group 60 to 69, and 5) 7.6 percent in age group 70 and older.

Of the 3,752 men who are classified as ex-regular cigarette smokers in table 2, 1,297 (34.6%) said that they gave up cigarette smoking less than 5 years prior to the time they answered the questionnaire.

Age at Which Cigarette Smoking Was Begun

Table 3 shows the ages at which current regular cigarette smokers said they began to smoke cigarettes. Among men, the mean age at start of cigarette smoking increased somewhat with present age, rising from 18.3 in present age group 30 to 39 to 24.2 in present age group 70 and older. Among females, this trend is much greater, rising from 20.2 in present age group 30 to 39 to 42.2 in present age group 70 and older. Particularly in the older groups a very much larger percentage of female cigarette smokers than male cigarette smokers took up the habit relatively late in life.

Current Cigarette Smoking

In table 4, the current regular cigarette smokers are classified by number of cigarettes smoked per day. A very small percentage of men are classified as amount "uncertain." Few women fell in this category, and they were included in the "uncertain" category in table 1 and are omitted from table 4.

The proportion of heavy cigarette smokers in the population decreased with age and was much greater among men than among women. For example, the percentage of people who currently smoked 20 or more cigarettes per day was 45.2 percent for men and 19.7 percent for women in age group 30 to 39 and 8.7 percent for men and 1.5 percent for women in age group 70 and older. The same data may be viewed in another way. In regard to people who were only current regular cigarette smokers the percentage smoking 20 or more cigarettes per day was 76.3 percent for men and 47.2 percent for women in age group 30 to 39 and 41.9 percent for men and 23.9 percent for women in age group 70 and older. Not only is cigarette smoking more common in men than in women, but males tend to smoke many more cigarettes per day than do females.

In table 5, men who currently smoked cigarettes regularly are divided into two groups: 1) those who smoked only cigarettes, and 2) those who smoked cigars or pipes as well as cigarettes. The former group tended to smoke more cigarettes per day than the latter group.

Nicotine and Tar

Cigarette smokers were asked what brand of cigarettes they smoked and whether they smoked filter-tip or nonfilter-tip cigarettes. Data on the nicotine and tar content of the main-stream smoke of popular brand cigarettes sold in 1959 (shortly before the start of our study) were deter-

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TABLE 3.—Age at which cigarette smoking was begun, for current regular cigarette smokers by age and sex

Age at which cigarette smoking was begun	Males										Females									
	Age 30–39		Age 40–49		Age 50–59		Age 60–69		Age 70+		Age 30–39		Age 40–49		Age 50–59		Age 60–69		Age 70+	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Total*	684	100.0	2,809	100.0	3,297	100.0	1,325	100.0	287	100.0	947	100.0	2,867	100.0	1,998	100.0	551	100.0	124	100.0
Under 14	82	12.0	352	12.5	389	11.8	196	14.8	48	16.7	36	3.8	91	3.2	23	1.2	4	0.7	—	—
15–19	440	64.3	1,609	57.3	1,781	54.0	612	46.2	100	34.8	541	57.1	1,256	43.8	383	19.2	37	6.7	6	4.8
20–24	139	20.3	638	22.7	804	24.4	312	23.5	59	20.6	243	25.7	849	29.6	544	27.2	52	9.4	6	4.8
25–29	18	2.6	127	4.5	174	5.2	90	6.8	25	8.7	78	8.2	300	10.5	374	18.7	83	15.1	11	8.9
30–34	3	0.4	38	1.4	65	2.0	40	3.0	11	3.8	41	4.3	172	6.0	264	13.2	105	19.1	12	9.7
35–39	2	0.3	19	0.7	29	0.9	25	1.9	3	1.0	8	0.8	111	3.9	164	8.2	104	18.9	16	12.9
40–44	—	—	21	0.7	29	0.9	23	1.7	13	4.5	—	—	68	2.4	118	5.9	68	12.3	18	14.5
45–49	—	—	5	0.2	14	0.4	10	0.8	4	1.4	—	—	20	0.7	77	3.9	29	5.3	22	17.7
50+	—	—	—	—	12	0.4	17	1.3	24	8.4	—	—	—	—	51	2.6	69	12.5	33	26.6
Mean age began		18.3		19.0		19.6		20.5		24.2		20.2		22.3		27.8		35.4		42.2

*Subjects who did not specify age they began smoking are omitted from this table.

TABLE 4.—Current regular cigarette smoking by number of cigarettes per day and by age and sex

Current number of cigarettes per day	Total		Age 30–39		Age 40–49		Age 50–59		Age 60–69		Age 70+	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Males:												
None or occasional*	9, 963	53. 3	484	40. 7	2, 357	44. 8	3, 466	50. 4	2, 433	63. 6	1, 223	79. 2
Total, regular cigarette	8, 734	46. 7	705	59. 3	2, 903	55. 2	3, 413	49. 6	1, 391	36. 4	322	20. 8
1–9	1, 015	5. 4	69	5. 8	261	5. 0	356	5. 2	222	5. 8	107	6. 9
10–19	1, 648	8. 8	95	8. 0	497	9. 4	626	9. 1	355	9. 3	75	4. 9
20	3, 243	17. 3	298	25. 1	1, 033	19. 6	1, 266	18. 4	538	14. 1	108	7. 0
21–39	1, 725	9. 2	165	13. 9	694	13. 2	694	10. 1	157	4. 1	15	1. 0
40	857	4. 6	59	5. 0	314	6. 0	379	5. 5	93	2. 4	12	0. 8
41+	184	1. 0	16	1. 3	89	1. 7	67	1. 0	12	0. 3	0	0
Uncertain	62	0. 3	3	0. 3	15	0. 3	25	0. 4	14	0. 4	5	0. 3
Total	18, 697	100. 0	1, 189	100. 0	5, 260	100. 0	6, 879	100. 0	3, 824	100. 0	1, 545	100. 0
Females:												
None or occasional*	17, 727	72. 7	1, 357	58. 3	4, 894	62. 6	5, 693	73. 6	3, 850	87. 0	1, 933	93. 5
Total, regular cigarette	6, 644	27. 3	970	41. 7	2, 920	37. 4	2, 046	26. 4	574	13. 0	134	6. 5
1–9	1, 720	7. 1	218	9. 4	668	8. 5	555	7. 2	217	4. 9	62	3. 0
10–19	2, 005	8. 2	294	12. 6	896	11. 5	622	8. 0	153	3. 5	40	1. 9
20	2, 098	8. 6	321	13. 8	955	12. 2	637	8. 2	156	3. 5	29	1. 4
21–39	594	2. 4	101	4. 3	302	3. 9	156	2. 0	33	0. 7	2	0. 1
40	207	0. 8	34	1. 5	87	1. 1	70	0. 9	15	0. 3	1	<0. 1
41+	20	0. 1	2	0. 1	12	0. 2	6	0. 1	0	0	0	0
Total	24, 271	100. 0	2, 327	100. 0	7, 814	100. 0	7, 739	100. 0	4, 424	100. 0	2, 067	100. 0

*People who currently do not smoke cigarettes regularly, some of whom are ex-regular cigarette smokers.

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TABLE 5.—Men who were current regular cigarette smokers according to current smoking pattern, by age, and current number of cigarettes per day

Current number of cigarettes per day	Total		Age 30–39		Age 40–49		Age 50–59		Age 60–69		Age 70+	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Smoke cigarettes only:												
1–9	687	9.2	44	7.2	174	6.9	245	8.2	142	12.5	82	30.7
10–19	1,336	17.8	80	13.1	419	16.6	509	17.1	267	23.5	61	22.8
20	2,865	38.2	262	43.0	899	35.7	1,133	38.1	475	41.8	96	36.0
21–39	1,591	21.2	155	25.5	640	25.4	639	21.5	143	12.6	14	5.2
40	795	10.6	51	8.4	291	11.5	357	12.0	84	7.4	12	4.5
41+	172	2.3	14	2.3	82	3.3	65	2.2	11	1.0	0	0
Uncertain	55	0.7	3	0.5	15	0.6	22	0.7	13	1.1	2	0.7
Total	7,501	100.0	609	100.0	2,520	100.0	2,970	100.0	1,135	100.0	267	100.0
Smoke cigarettes and cigars or pipes:												
1–9	328	26.6	25	26.0	87	22.7	111	25.1	80	31.2	25	45.5
10–19	312	25.3	15	15.6	78	20.4	117	26.4	88	34.4	14	25.5
20	378	30.7	36	37.5	134	35.0	133	30.0	63	24.6	12	21.8
21–39	134	10.9	10	10.4	54	14.1	55	12.4	14	5.5	1	1.8
40	62	5.0	8	8.3	23	6.0	22	5.0	9	3.5	0	0
41+	12	1.0	2	2.1	7	1.8	2	0.5	1	0.4	0	0
Uncertain	7	0.6	0	0	0	0	3	0.7	1	0.4	3	5.5
Total	1,233	100.0	96	100.0	383	100.0	443	100.0	256	100.0	55	100.0

TABLE 6.—Current regular cigarette smokers by type of cigarette, nicotine content, age, and sex

Type of cigarette and nicotine content	Total		Age 30–39		Age 40–49		Age 50–59		Age 60–69		Age 70+	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Males:												
Type:												
Filter-tip	4, 308	49. 3	357	50. 6	1, 396	48. 1	1, 706	50. 0	684	49. 2	165	51. 2
Nonfilter	4, 007	45. 9	319	45. 2	1, 393	48. 0	1, 535	45. 0	619	44. 5	141	43. 8
Type unknown*	419	4. 8	29	4. 1	114	3. 9	172	5. 0	88	6. 3	16	5. 0
Nicotine content:												
0.4–1.1 mg	1, 011	11. 6	97	13. 8	334	11. 5	398	11. 7	151	10. 9	31	9. 6
1.2–1.8 mg	2, 902	33. 2	258	36. 6	976	33. 6	1, 102	32. 3	460	33. 1	106	32. 9
1.9–2.7 mg	3, 626	41. 5	273	38. 7	1, 256	43. 3	1, 402	41. 1	556	40. 0	139	43. 2
Unknown†	1, 195	13. 7	77	10. 9	337	11. 6	511	15. 0	224	16. 1	46	14. 3
Total number	8, 734	100. 0	705	100. 0	2, 903	100. 0	3, 413	100. 0	1, 391	100. 0	322	100. 0
Females:												
Type:												
Filter-tip	4, 776	71. 9	659	67. 9	2, 025	69. 3	1, 535	75. 0	455	79. 3	102	76. 1
Nonfilter	1, 731	26. 1	296	30. 5	829	28. 4	465	22. 7	110	19. 2	31	23. 1
Type unknown*	137	2. 1	15	1. 5	66	2. 3	46	2. 2	9	1. 6	1	0. 7
Nicotine content:												
0.4–1.1 mg	1, 479	22. 3	195	20. 1	615	21. 1	476	23. 3	160	27. 9	33	24. 6
1.2–1.8 mg	2, 282	34. 3	340	35. 1	1, 019	34. 9	694	33. 9	180	31. 4	49	36. 6
1.9–2.7 mg	2, 122	31. 9	352	36. 3	972	33. 3	599	29. 3	161	28. 0	38	28. 4
Unknown†	761	11. 5	83	8. 6	314	10. 8	277	13. 5	73	12. 7	14	10. 4
Total number	6, 644	100. 0	970	100. 0	2, 920	100. 0	2, 046	100. 0	574	100. 0	134	100. 0

*Type not stated or both filter-tip and nonfilter-tip cigarettes.
†Brand not stated or two or more different brands.

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mined by Foster D. Snell, Inc., consulting chemists and engineers, for the *Reader's Digest* and were published in the November, 1959, issue in an article by Lois Mattox Miller and James Monahan (*4*). With this data, we were able to classify the subjects according to the tar and nicotine content of the cigarettes they usually smoked. The results are summarized in table 6 in terms of nicotine content. Tar and nicotine content are closely related. With a few minor exceptions, cigarettes low in nicotine are also low in tar and vice versa.

Filter-tip cigarettes are more popular among female than male cigarette smokers. More important, female smokers tend to smoke cigarettes far lower in nicotine and tar content than those of the average male smoker. About twice as large a proportion of female as male smokers consumed cigarettes having less than 1.2 mg of nicotine in the main-stream smoke. About the same proportion of men and women smoked cigarettes in the 1.2 to 1.8 mg category. Proportionally fewer women than men smoked cigarettes having 1.9 to 2.7 mg of nicotine in the main-stream smoke.

In informal discussions it has sometimes been suggested that lowering the tar and nicotine content of cigarettes would do no good since smokers might make up for it by smoking more cigarettes per day. So far as we know, there is no solid evidence in support of this hypothesis. Table 7 shows the percentage distribution by number of cigarettes smoked per day for current regular cigarette smokers who smoked only cigarettes; these persons are divided into three groups, by nicotine content of the main-stream smoke of the cigarettes they consumed. The data for males have been age standardized to the age distribution of all male regular cigarette smokers in the study. The data for females have been age standardized to the age distribution of all female regular cigarette smokers in the study.

TABLE 7.—Percentage distribution of amount of current regular cigarette smoking only by three levels of nicotine content* by sex, standardized for age

Current number of cigarettes per day	0.4–1.1 mg	1.2–1.8 mg	1.9–2.7 mg
Male:			
1–9	11.4	9.0	7.9
10–19	16.0	18.3	17.5
20	31.5	38.3	41.4
21–39	29.5	21.3	21.0
40+	15.2	13.2	12.2
Total	100.0	100.0	100.0
Female:			
1–9	30.2	24.9	22.2
10–19	29.8	30.2	31.1
20	27.1	33.4	34.5
21–39	9.3	9.1	8.3
40+	3.7	2.4	4.0
Total	100.0	100.0	100.0

*Persons who did not state their brand of cigarettes are omitted from this table.

The average number of cigarettes smoked per day does not appear to be significantly related to their nicotine content. However, the variance of the distribution by amount does vary somewhat in relation to nicotine content. In general, the pattern seems to be as follows: With increasing nicotine content there is a moderate increase in the proportion of people who smoked about 20 cigarettes a day and a corresponding decrease in both the proportion of people smoking very few cigarettes and the proportion smoking a very large number of cigarettes. This pattern is more consistent in the data for men than for women.

This evidence casts doubt on the hypothesis stated but does not necessarily disprove it. In 1959, low-nicotine cigarettes had been on the market only for a relatively short time. Most people then smoking these cigarettes had presumably smoked higher-nicotine cigarettes previously. Unfortunately, we have no information on the number of cigarettes smoked per day by these particular subjects before they made the switch. Personally, we doubt that the point at issue could be reliably answered by questioning low-nicotine cigarette smokers as to how many relatively high-nicotine cigarettes they had smoked per day before. In our opinion, it would be necessary to question a large number of individuals as to their current smoking habits and then ask them the same questions again a year or two later.

Inhalation

Smokers were asked whether they inhaled the smoke "none," "slightly," "moderately," or "deeply." The answers from males are summarized in table 8 by type of smoking. In this table, the degree of inhalation of cigarette smoke is recorded for men who smoked cigarettes and also pipes or cigars, since it is extremely rare for such a man to say that he inhales cigar or pipe smoke to a greater degree than he inhales cigarette smoke; (however, many men with mixed habits said that they inhaled cigarette smoke much more than they inhaled cigar or pipe smoke).

Briefly stated, men who smoke only cigarettes usually inhale moderately or deeply; men who smoke only pipes usually do not inhale or inhale slightly; and men who smoke only cigars inhale less than men who smoke only pipes. Men who smoke cigarettes and pipes inhale somewhat less

TABLE 8.—Percentage distribution of degree of inhalation* by current type of smoking for males, standardized for age†

Current type of smoking	Total	Do not inhale	Inhale slightly	Inhale moderately	Inhale deeply
Cigarette only	100.0	7.1	14.4	54.6	23.9
Cigarette and pipe	100.0	12.3	16.9	52.4	18.4
Cigarette and cigar	100.0	24.7	24.3	34.6	16.4
Cigarette, cigar, and pipe	100.0	32.0	24.2	34.5	9.4
Pipe only	100.0	53.3	24.0	19.8	2.9
Cigar only	100.0	70.6	19.6	8.3	1.5

*Persons who did not state the degree of inhalation are omitted from this table.
†Men with mixed smoking habits are classified according to the degree to which they inhale cigarette smoke.

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TABLE 9.—Amount of inhalation* of cigarette smoke by age for males and females

	Total		Do not inhale		Inhale slightly		Inhale moderately		Inhale deeply	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Age 30–39:										
Male, cigarette only	602	100.0	16	2.7	59	9.8	311	51.7	216	35.9
Male, cigarette and other	95	100.0	16	16.8	18	18.9	40	42.1	21	22.1
Female	964	100.0	54	5.6	174	18.0	563	58.4	173	17.9
Age 40–49:										
Male, cigarette only	2,498	100.0	90	3.6	278	11.1	1,465	58.6	665	26.6
Male, cigarette and other	381	100.0	51	13.4	68	17.8	174	45.7	88	23.1
Female	2,915	100.0	238	8.2	637	21.9	1,624	55.7	416	14.3
Age 50–59:										
Male, cigarette only	2,940	100.0	186	6.3	378	12.9	1,651	56.2	725	24.7
Male, cigarette and other	440	100.0	98	22.3	87	19.8	192	43.6	63	14.3
Female	2,034	100.0	369	18.1	546	26.8	907	44.6	212	10.4
Age 60+:										
Male, cigarette only	1,379	100.0	192	13.9	303	22.0	670	48.6	214	15.5
Male, cigarette and other	307	100.0	81	26.4	85	27.7	111	36.2	30	9.8
Female	699	100.0	253	36.2	220	31.5	190	27.2	36	5.2

*Persons who did not state degree of inhalation are omitted from this table.

with cigarettes than do men who smoke only cigarettes. Men who smoke both cigarettes and cigars inhale less with cigarettes than do men who smoke cigarettes and pipes.

Table 9 shows degree of inhalation of cigarette smoke by age and sex. Within the same age groups, men tend to inhale cigarette smoke to a much greater degree than do women. In both men and women degree of inhalation of cigarettes decreases with age.

As shown in table 10, degree of inhalation of cigarette smoke increases markedly with number of cigarettes smoked per day. Similar findings on inhalation in relation to type and amount of smoking and age have been previously reported for men in the United States (*5*) and for both men and women in Great Britain (*6*).

TABLE 10.—Percent distribution of amount of current regular cigarette smoking by inhalation by sex, standardized for age*

Current number of cigarettes per day	Total	Do not inhale	Inhale slightly	Inhale moderately	Inhale deeply
Males:					
1–9	100.0	20.2	27.9	45.0	6.8
10–19	100.0	6.7	17.3	62.0	14.0
20	100.0	5.4	12.9	59.9	21.8
21–39	100.0	3.8	9.1	51.3	35.9
40+	100.0	5.2	9.5	45.7	39.5
Females:					
1–9	100.0	29.7	36.1	31.2	2.9
10–19	100.0	9.4	25.8	55.9	8.9
20	100.0	6.9	17.9	58.3	17.0
21–39	100.0	6.8	11.8	53.7	27.8
40+	100.0	8.0	10.8	45.2	36.0

*Persons who did not state degree of inhalation are omitted from this table. In this table, the data for males are confined to men who smoked only cigarettes.

Nicotine and Inhalation

One might have suspected that smoke from low-nicotine cigarettes would be inhaled to a greater degree than smoke from high-nicotine cigarettes. Table 11 was prepared to obtain some evidence on this point. Men with mixed smoking habits are excluded from this table. The data for males are standardized for age to the age distribution of the male cigarette smokers. The data for females are standardized for age to the age distribution of the female cigarette smokers.

The following comparisons were made among males who smoked the same number of cigarettes per day: Men who smoked cigarettes with less than 1.2 mg of nicotine said that they inhaled to a lesser degree, on the average, than did men who smoked cigarettes with 1.2 to 1.8 mg of nicotine; men who smoked cigarettes in the 1.2 to 1.8 mg category said that they inhaled a bit less on the average than did those who smoked cigarettes with 1.9 to 2.7 mg of nicotine.

Among women, there appears to be little or no relationship between degree of inhalation and nicotine content of the smoke.

TABLE 11.—Percentage distribution of degree of inhalation of cigarette smoke by nicotine content, amount of cigarette smoking, and sex, standardized for age*

Nicotine content	Current number of cigarettes per day	Males					Females				
		Total percent	Do not inhale	Inhale slightly	Inhale moderately	Inhale deeply	Total percent	Do not inhale	Inhale slightly	Inhale moderately	Inhale deeply
0.4–1.1 mg nicotine	1–9	100. 0	30. 0	25. 4	41. 1	3. 4	100. 0	31. 7	33. 0	33. 1	2. 2
	10–19	100. 0	10. 5	17. 4	61. 5	10. 5	100. 0	8. 6	27. 5	55. 6	8. 4
	20	100. 0	5. 9	18. 3	58. 0	17. 8	100. 0	5. 6	16. 9	63. 0	14. 4
	21–39	100. 0	2. 1	7. 5	57. 5	32. 9	100. 0	5. 5	17. 3	56. 7	20. 5
	40+	100. 0	5. 8	12. 6	42. 8	38. 9	100. 0†	8. 6	8. 9	34. 2	48. 3
1.2–1.8 mg nicotine	1–9	100. 0	20. 5	30. 2	42. 9	6. 4	100. 0	32. 0	37. 2	28. 6	2. 2
	10–19	100. 0	6. 4	19. 1	60. 6	13. 8	100. 0	9. 7	24. 3	56. 4	9. 5
	20	100. 0	4. 9	11. 6	61. 7	21. 8	100. 0	6. 5	17. 9	58. 9	16. 7
	21–39	100. 0	2. 5	8. 0	53. 8	35. 6	100. 0	4. 0	10. 7	53. 8	31. 6
	40+	100. 0	3. 2	10. 3	48. 5	38. 0	100. 0†	10. 5	10. 7	50. 8	28. 0
1.9–2.7 mg nicotine	1–9	100. 0	16. 3	32. 0	43. 6	8. 0	100. 0	32. 1	32. 0	32. 0	3. 8
	10–19	100. 0	6. 3	17. 1	61. 4	15. 2	100. 0	10. 1	25. 1	55. 3	9. 5
	20	100. 0	5. 3	12. 5	58. 6	23. 6	100. 0	6. 7	16. 6	57. 6	19. 1
	21–39	100. 0	3. 3	8. 4	49. 4	38. 8	100. 0	6. 4	9. 7	51. 4	32. 5
	40+	100. 0	4. 0	7. 2	47. 2	41. 6	100. 0†	4. 6	12. 3	49. 6	33. 5

*In this table, the data for males are confined to men who smoked only cigarettes.
†Because only a small number of women smoked 40+ cigarettes per day, the percentages on these lines are rather unstable statistically.

Education

In table 12 the subjects are divided into the following 5 groups by education: 1) no high school, 2) some high school, 3) high school graduate, 4) some college, and 5) college graduate. The total number of subjects, the percentage who currently smoked cigarettes regularly, and the percentage who currently smoked 21 or more cigarettes per day are shown for each of the education groups by age. Also shown are the number of subjects with a history of regular cigarette smoking and the percentage of these who had given up the habit, (*i.e.*, ex-regular cigarette smokers).

In males, the lowest percentage of current regular cigarette smokers was in the college-graduate group and the highest in the some-high-school group. The college-graduate and the no-high-school group had lower percentages of heavy cigarette smokers than other groups.

In females, the college-graduate group and the no-high-school group tended to have a lower percentage of cigarette smokers than the other groups. However, the differences were not very great.

The percentage of ex-regular cigarette smokers (in relation to the number of subjects with a history of regular cigarette smoking) was higher in the college-graduate group than in any educational group. This was found in both males and females in every age group.

Table 13 shows current regular cigarette smokers (regardless of other types of smoking) classified according to the nicotine content of the cigarette they usually smoked. The percentage of men smoking cigarettes with less than 1.2 mg of nicotine increased with amount of education. The difference in this respect between extreme groups (*i.e.*, no high school versus college graduate) was considerable in every age group. The same general pattern was found in females but it was not quite as consistent as in males.

Occupation

In table 14 the men are divided into the following 4 broad occupational groups: 1) farmers, including some who also had other occupations; 2) teachers, members of the legal profession, and clergymen; 3) doctors, dentists, and veterinarians; and 4) all other occupations. These particular groups were selected for the following reasons: 1) Death rates from lung cancer and a number of other diseases are reported to be lower in rural areas than in urban areas; 2) doctors, veterinarians, and dentists have a professional interest in health; and 3) teachers, members of the legal profession, and clergymen (because of their position in society) have perhaps taken more interest in the problem of smoking in relation to health than has the public in general.

The percentages of current regular cigarette smokers were lower in all 3 of the specified occupational groups than in the remainder of the subjects. This was found in every age group.

The percentages of men smoking 21 or more cigarettes per day were substantially lower among farmers and teachers, members of the legal profession, and clergymen than among doctors, dentists, veterinarians,

TABLE 12.—Current regular cigarette smoking and ex-cigarette smoking groups by education,* by age and sex

	Males					Females				
	No high school	Some high school	High school graduate	Some college	College graduate	No high school	Some high school	High school graduate	Some college	College graduate
Age 30–39:										
Number of subjects	99	185	324	228	341	98	377	994	501	347
Percent current cigarette	61.6	67.0	58.6	62.7	52.5	39.8	46.4	40.1	44.1	38.6
Percent 21+ cigarettes	18.2	25.9	20.4	24.1	15.2	2.0	4.8	5.9	7.2	6.3
Number with history of regular cigarette	73	150	244	171	232	41	200	464	266	163
Percent ex-cigarette	16.4	16.7	21.3	16.4	22.8	4.9	12.5	14.0	16.9	17.8
Age 40–49:										
Number of subjects	688	979	1,384	1,057	1,117	713	1,439	2,645	1,802	1,173
Percent current cigarette	57.0	62.1	56.1	57.2	43.9	21.2	40.9	36.1	44.3	35.3
Percent 21+ cigarettes	13.1	22.2	22.0	25.8	18.5	2.2	6.3	4.6	7.2	3.8
Number with history of regular cigarette	508	784	1,021	810	729	185	666	1,110	961	536
Percent ex-cigarette	22.0	22.3	23.6	24.9	32.2	18.4	11.7	13.9	16.9	22.8
Age 50–59:										
Number of subjects	1,535	1,491	1,145	1,276	1,379	1,282	1,597	1,658	1,856	1,294
Percent current cigarette	49.4	54.4	51.9	49.7	40.8	17.5	26.4	29.1	29.2	28.7
Percent 21+ cigarettes	12.3	16.6	18.3	20.8	16.2	1.0	2.3	3.9	3.8	3.8
Number with history of regular cigarette	1,046	1,106	813	919	910	264	489	563	658	658
Percent ex-cigarette	26.6	26.1	26.8	30.4	38.0	15.2	13.7	14.2	14.2	17.6
Age 60+:										
Number of subjects	2,153	1,088	504	771	727	2,113	1,458	948	1,135	710
Percent current cigarette	30.7	33.3	32.3	36.6	26.3	7.4	12.5	14.6	12.4	12.3
Percent 21+ cigarettes	4.0	5.4	6.3	8.8	5.6	0.3	0.8	1.2	1.5	0.7
Number with history of regular cigarette	1,103	633	288	470	351	203	237	163	182	118
Percent ex-cigarette	39.0	42.2	43.1	40.0	45.3	22.7	23.2	15.3	22.5	26.3

*Persons who did not state their education are omitted from this table.

TABLE 13.—Current regular cigarette smokers according to nicotine content, by education,* by age and sex

Age group	Nicotine content†	Males										Females									
		No high school		Some high school		High school graduate		Some college		College graduate		No high school		Some high school		High school graduate		Some college		College graduate	
		Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
30-39	<1.2 mg	3	5.3	12	10.7	22	13.1	17	13.3	43	27.2	3	9.4	21	12.7	83	22.8	59	29.5	29	23.6
	1.2-1.8 mg	25	43.9	50	44.6	70	41.7	49	38.3	62	39.2	18	56.3	65	39.2	149	40.9	63	31.5	44	35.8
	1.9+ mg	29	50.9	50	44.6	76	45.2	62	48.4	53	33.5	11	34.4	80	48.2	132	36.3	78	39.0	50	40.7
	Total	57		112		168		128		158		32		166		364		200		123	
40-49	<1.2 mg	22	6.3	47	8.8	68	9.8	87	16.0	109	25.4	18	13.3	100	19.1	192	22.4	195	27.5	108	29.3
	1.2-1.8 mg	135	38.6	190	35.6	271	39.1	214	39.3	162	37.8	56	41.5	195	37.2	361	42.0	249	35.2	151	41.0
	1.9+ mg	193	55.1	297	55.6	354	51.1	244	44.8	158	36.8	61	45.2	229	43.7	306	35.6	264	37.3	109	29.6
	Total	350		534		693		545		429		135		524		859		708		368	
50-59	<1.2 mg	49	7.4	83	12.0	62	12.3	83	15.1	119	25.0	33	16.1	70	19.8	120	28.4	139	29.8	114	35.7
	1.2-1.8 mg	236	35.9	268	38.8	204	40.5	207	37.7	175	36.8	90	43.9	143	40.5	168	39.8	187	40.0	104	32.6
	1.9+ mg	373	56.7	339	49.1	238	47.2	259	47.2	182	38.2	82	40.0	140	39.7	134	31.8	141	30.2	101	31.7
	Total	658		690		504		549		476		205		353		422		467		319	
60+	<1.2 mg	48	8.6	36	11.7	26	18.4	37	15.2	35	21.3	34	24.5	44	27.7	41	34.5	48	37.5	26	35.1
	1.2-1.8 mg	224	40.2	111	36.0	51	36.2	107	43.9	58	35.4	59	42.4	58	36.5	34	28.6	51	39.8	26	35.1
	1.9+ mg	285	51.2	161	52.3	64	45.4	100	41.0	71	43.3	46	33.1	57	35.8	44	37.0	29	22.7	22	29.7
	Total	557		308		141		244		164		139		159		119		128		74	

*Persons who did not state their education are omitted from this table.
†Persons who did not state their brand of cigarettes are omitted from this table.

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TABLE 14.—Current regular cigarette smoking and ex-cigarette smoking by occupation and age for males

	Farmer	Teacher, legal profession, and clergy	Doctor, dentist, and veterinarian	All other
Age 30–39:				
Number of subjects	62	76	22	1,029
Percent current cigarette	48.4	46.1	50.0	60.8
Percent 21+ cigarettes	12.9	6.6	22.7	21.6
Number with history of regular cigarette	40	52	14	771
Percent ex-cigarette	25.0	32.7	21.4	18.4
Age 40–49:				
Number of subjects	426	284	109	4,441
Percent current cigarette	47.7	40.1	35.8	57.0
Percent 21+ cigarettes	13.6	13.0	16.5	22.2
Number with history of regular cigarette	272	166	70	3,368
Percent ex-cigarette	25.0	30.1	41.4	24.5
Age 50–59:				
Number of subjects	666	397	118	5,698
Percent current cigarette	34.7	33.2	45.8	52.1
Percent 21+ cigarettes	9.0	8.8	18.6	18.0
Number with history of regular cigarette	346	217	84	4,182
Percent ex-cigarette	32.4	39.2	35.7	28.4
Age 60+:				
Number of subjects	904	224	74	4,167
Percent current cigarette	24.2	19.2	28.4	33.9
Percent 21+ cigarettes	2.4	2.2	8.1	6.1
Number with history of regular cigarette	357	79	37	2,431
Percent ex-cigarette	36.7	45.6	43.2	41.5

and the "all-other" group. However, the percentage of men smoking 21 or more cigarettes per day did not differ much between the doctor-dentist-veterinarian group and the "all-other" group.

The percentages of ex-regular cigarette smokers (in relation to the number of subjects with a history of regular cigarette smoking) were higher in the teacher-legal-clergy and the doctor-dentist-veterinarian group than in other groups.

Table 15 shows current regular cigarette smokers (regardless of other types of smoking) classified according to the nicotine content of the cigarettes they usually smoke. The data have been age standardized to the age distribution of all men in the study who were current regular cigarette smokers.

TABLE 15.—Percentage distribution of current regular cigarette smokers by nicotine content by occupation for males, standardized for age

Nicotine content*	Farmer	Teacher, legal profession, and clergy	Doctor, dentist, and veterinarian	All other
<1.2 mg	7.2	21.1	23.5	13.4
1.2–1.8 mg	41.6	36.4	39.8	38.1
1.9+ mg	51.1	42.4	36.8	48.4
Total	100.0	100.0	100.0	100.0

*Persons who did not state their brand of cigarettes are omitted from this table.

The teacher-legal-clergy and the doctor-dentist-veterinarian group showed a much higher percentage of smokers who currently used cigarettes with less than 1.2 mg of nicotine than was found in the farmer group and in the "all-other" group. Farmers showed the lowest percentage in the under 1.2 mg of nicotine category.

Marital Status

The subjects for this study were enrolled in family groups (*i.e.*, households). Although by our definition a family can consist of just one person living by himself, a large proportion of the families contained a married couple. In this sample, 76.5 percent of the women and 94.4 percent of the men were married.

The proportion of women who were current regular cigarette smokers and the proportion who currently smoked 20 or more cigarettes per day are shown by marital status and age in table 16. Within each age group, the proportion of current regular cigarette smokers was lowest among single women and highest among women who were divorced or separated. The proportion of regular cigarette smokers was somewhat higher among widows than among married women.

TABLE 16.—Number and percent of current regular cigarette smokers by marital status* and age for females

	Single		Married		Widowed		Divorced or separated	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Age 30–39:								
Number of women*	209	100.0	1,968	100.0	28	100.0	101	100.0
Current cigarette	77	36.8	815	41.4	12	42.9	64	63.4
20+ Cigarettes	37	17.7	386	19.6	7	25.0	27	26.7
Age 40–49:								
Number of women*	301	100.0	7,040	100.0	192	100.0	217	100.0
Current cigarette	93	30.9	2,617	37.2	75	39.1	128	59.0
20+ Cigarettes	36	12.0	1,227	17.4	32	16.7	61	28.1
Age 50–59:								
Number of women*	418	100.0	6,278	100.0	736	100.0	238	100.0
Current cigarette	103	24.6	1,611	25.7	238	32.3	92	38.7
20+ Cigarettes	32	7.7	697	11.1	99	13.5	41	17.2
Age 60–69:								
Number of women*	300	100.0	2,761	100.0	1,156	100.0	159	100.0
Current cigarette	29	9.7	310	11.2	184	15.9	49	30.8
20+ Cigarettes	9	3.0	114	4.1	62	5.4	18	11.3
Age 70+:								
Number of women*	177	100.0	608	100.0	1,231	100.0	35	100.0
Current cigarette	7	4.0	36	5.9	87	7.1	4	11.4
20+ Cigarettes	1	0.6	10	1.6	20	1.6	1	2.9

*Women who did not state their marital status are excluded from this table.

Of the men, only 395 were single, 361 widowed, and 137 divorced or separated. These were distributed by age in such a way that comparisons of their smoking habits within age groups were very unstable statistically because of small numbers. Therefore, although the pattern of cigarette

smoking in relation to marital status appeared to be about the same among men as women, this cannot be regarded as a definite finding.

DISCUSSION

The following tobacco-use variables have been studied both singly and in combination: 1) current type of tobacco use (*i.e.*, none, cigarette, cigar, pipe, and chewing tobacco and snuff); 2) past smoking of current non-smokers; 3) degree of inhalation of tobacco smoke; 4) current number of cigarettes smoked per day; 5) type of cigarette (*i.e.*, filter-tip or non-filter-tip); 6) nicotine and tar content of main-stream smoke of cigarettes; 7) ages at which current cigarette smokers began to smoke cigarettes; and 8) number of years since ex-cigarette smokers last smoked cigarettes.

All these variables are related to the degree and type of exposure of individuals to tobacco or tobacco-smoke products.

The findings indicate that exposure to cigarette-smoke products is far less in the female population than in the male population. For example:

1) With type of smoking disregarded, the proportion of current smokers is much smaller among women than men.
2) The proportion of current regular cigarette smokers is much smaller among women than men.
3) Among current regular cigarette smokers, the proportion of heavy cigarette smokers is much smaller among women than men.
4) Among current regular cigarette smokers, the average age at start of cigarette smoking is older among women than men (*i.e.*, within the same age groups, the average female cigarette smoker has been smoking for less time than has the average male cigarette smoker).
5) With age and current amount of cigarette smoking held constant, women tend to inhale cigarette smoke to a lesser degree than do men.
6) Among current regular cigarette smokers, a far larger proportion of women than men smoke cigarettes with a very low concentration of nicotine and tar in the main-stream smoke.

These differences between men and women increased with age, the largest relative differences being found in the older age groups.

In our opinion, all the exposure factors described should be taken into consideration in comparative studies between men and women in respect to the association between smoking and various diseases. For example, if some disease is highly associated with degree of exposure to cigarette-smoke products (and sex *per se* is unrelated to the disease), then one would expect to find a higher incidence of the disease among male cigarette smokers than among female cigarette smokers even if current number of cigarettes smoked per day is held constant.

The subjects for this study were enrolled in family (*i.e.*, household) groups. Thus the men and women were largely from the same families, in a large proportion husband and wife. Therefore, to a considerable extent the men and women were matched for such factors as residence,

economic status, social status, race, and religion. We consider this to be an asset from the standpoint of comparing the smoking habits of men with those of women.

Among men in all year-of-birth cohorts, the great majority of current regular smokers had taken up the habit before reaching the age of 30, while the proportion of ex-smokers increased with age. This partially accounts for the decline in the proportion of current regular smokers with advancing age. However, the fact that male smokers (particularly cigarette smokers) have a higher death rate than nonsmokers must have made a substantial contribution to the decline in the proportion of regular smokers with advancing age (*7*, *8*).

Among women, many current regular smokers in the older age groups took up smoking relatively late in life. In spite of this, the proportion of current regular smokers is considerably less among women in the older age groups than in the younger age groups. This must be largely due to changes in social customs during the last several decades, though it is partially because of some female smokers giving up the habit later in life. As yet, there is no firm evidence concerning the death rate of female smokers compared with female nonsmokers, so the possible effect of selective mortality cannot be evaluated at the present time.

Among men, particularly in the older age groups, an appreciable number of nonsmokers as well as smokers said that they chewed tobacco or used snuff. This may be of some importance in studies of tobacco use in respect to certain diseases such as cancer of the buccal cavity.

Since smoking habits are found to vary somewhat with education, occupation, and residence (*i.e.*, urban versus rural) it is desirable to take these factors into consideration in studies of smoking in relation to disease.

In this and earlier studies it has been found that cigar and pipe smokers tend to inhale tobacco smoke far less than do cigarette smokers. This may be the reason that a much higher association has been found (*7*, *8*) between cigarette smoking and death rates from certain diseases than between pipe and cigar smoking and death rates from these same diseases.

During the last few years, several brands of cigarettes have been put on the market which have far less nicotine and tar in the main-stream smoke than do brands that have been popular for many decades. Some people have suggested that this would do no good. They postulate that smokers who switched to these brands might smoke more cigarettes per day than they did formerly (so they would not materially reduce their daily intake of tar and nicotine).

Evidence from this study does not support the hypothesis that smokers who switch to low-tar, low-nicotine cigarettes tend to increase their daily consumption of cigarettes. However, the present evidence is of such a nature that it does not necessarily contradict the hypothesis.

Now let us assume for a moment that smokers who switch to low-tar, low-nicotine cigarettes actually do increase their daily consumption to such a degree that their daily intake of tar and nicotine remains unaltered (or may even be somewhat increased). Would this necessarily mean that,

from the standpoint of health, the switch to low-tar, low-nicotine cigarettes is of no advantage?

We think that this is not necessarily so for the following reasons: It is well known that if a large quantity of nicotine is administered in a single dose, it produces drastic acute effects; but if the same quantity of nicotine is administered in small doses spread over a period of many hours the acute effects are relatively very mild. The same is true of carbon monoxide and may be true of various other components of cigarette smoke. Possibly, the same sort of relationship between concentration and effect applies to the chronic effects of some components of tobacco smoke. In our opinion, this matter should be further investigated.

Acknowledgment

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Genetic and Environmental Influences on Teratocarcinogenesis in Mice [1, 2]

LEROY C. STEVENS *and* JUDITH A. MACKENSEN,[3]
Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

SUMMARY

Testicular teratomas occur in a predictable proportion of strain 129 mice. Subline differentiation has occurred within the strain, which has resulted in appreciable differences in the incidence of teratomas. The gene Steel (*Sl*) has been placed on the strain 129 background, and has markedly increased tumor incidence. The influence of the *Sl* gene upon teratocarcinogenesis in strain 129 mice is a new pleiotropic effect in addition to its other effects on the development of germ, pigment, and blood cells. Environmental factors also influence teratocarcinogenesis in mice. The incidence of teratomas in males of second and later litters is twice as great as in males of first litters, which suggests that the age of the mother may be a factor in tumor susceptibility. Teratomas occur more frequently in the left testis than the right, which indicates that the local environment also affects the initiation of teratocarcinogenesis. As a result of the information presented here, it has been feasible to undertake a study of the development of teratomas in fetal mice.—J. Nat. Cancer Inst. 27: 443–453, 1961.

TESTICULAR TERATOMAS characteristically occur in approximately 1 percent of male inbred strain 129 mice (*1*). The tumors are congenital, and they develop from nests of undifferentiated embryonal cells which form germ layers that give rise to many immature and adult tissues. Teratomas are easily recognized in testes of animals older than 4 to 5 days, but detection of tumors in younger mice requires microscopic examination of serial sections of whole testes. To study the development of tumors in prenatal mice it was desirable to develop a stock of animals in which an incidence higher than 1 percent could be expected. Other investigators have associated differences in tumor susceptibility with subline differentiation, action of specific genes, maternal influences, and local environmental influences within the body. These genetic and environmental modifying effects were investigated for their influence on susceptibility to teratocarcinogenesis in strain 129 mice.

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We examined several sublines of strain 129 mice in this laboratory to determine whether differences in teratoma incidence existed. Sublines of inbred mice, separated for many generations, may become different from each other in mammary-tumor incidence (*2*, *3*) and other characteristics (*4*); it is believed that gene mutation may be the basis for subline differentiation (*5*).

Single, specific genes have been associated with incidence of several types of tumors in mice (*6–9*). To determine the effect of single genes on testicular teratomas, we introduced several mutant genes, including Steel (*Sl*), onto the strain 129 genetic background, and their influence on the development of teratomas was observed. Steel was selected because it exerts striking effects upon the development of germ cells and has effects on pigment which makes its presence detectable (*10*).

The penetrance of various inherited characteristics has been observed to vary according to litter number or parental age (*11–13*). The effect of these on susceptibility to teratocarcinogenesis in strain 129 mice was analyzed. There is a strong position influence on teratocarcinogenesis in strain 129 mice: The tumor incidence in the left testis is over twice that of the right (*1*).

A stock of mice (129/Sv-*Sl*) was developed which has an incidence of spontaneous teratomas higher than 1 percent. Genetic (subline differentiation, introduction of the gene Steel) and environmental (litter seriation or maternal age) factors appreciably affected the incidence of tumors. Data supporting these statements are presented in this article.

MATERIALS

The incidences of teratomas in several sublines of inbred strain 129 mice were determined by postmortem examination of animals over 1 week of age when teratomas can be recognized grossly. Strain 129 mice were usually maintained with forced heterozygosity at the *C* locus, and breeding animals were of the genotype $c^{ch}cppslsl$.

A mutation occurring in C_3H/Hu is allelic to and indistinguishable from *Sl*, as described by Hummel and Russell, in Mouse News Letters No. 12, Feb., 1955, and No. 13, July, 1955. One mutant, an *Slsl* male, was obtained in 1954. It was mated with strain 129 females and the pigmented Steel offspring (*CcPpSlsl*) backcrossed repeatedly to strain 129. Backcrossing was continued for 12 to 14 generations and the new subline, designated 129/Sv-*Sl*, is considered coisogenic with 129/Sv except for alleles *C*, *P*, *Sl*, and possibly closely linked loci.

The effects of the *Sl* allele on pigmentation when combined with *C* and *P* were readily detectable by a lack of pigment in the tail tip. Tails of heavily pigmented (*C-P-slsl*) strain 129 mice were pigmented along their entire length. In animals lacking genes *C* and *P*, the presence of the *Sl* allele was not readily detectable except in animals with the genotype $c^{ch}c^{ch}P$-. We maintained the colony of 129/Sv-*Sl* mice by mating Steel

males (*C-P-Slsl* and $c^{ch}c^{ch}$*P-Slsl*) with Steel or non-Steel (*C-P-slsl* and $c^{ch}c^{ch}$*P-slsl*) females, not always sibs. Offspring were classified into three groups: 1) known-Steel (*C-P-Slsl* and $c^{ch}c^{ch}$*P-Slsl*), 2) recognized non-Steel (*C-P-slsl* and $c^{ch}c^{ch}$*P-slsl*), and 3) albino or pink-eyed animals (*cc*, $c^{ch}c$, $c^{ch}c^{ch}$, and *C-pp*), some of which carry the *Sl* allele.

Tumors were fixed in Vandegrift's solution, sectioned, and stained with hematoxylin and eosin.

RESULTS

Genetic Influences on Incidence of Testicular Teratomas

Subline differences.—Numerous males from each of several different sublines of inbred strain 129 mice were autopsied and examined for the presence of teratomas. Results are summarized in table 1. The incidence of teratomas ranged from 0.26 percent in the 129/Re to 1.70 percent in the 129/J subline. On the basis of these data, subline 129/J was selected as the source of a new colony, which was designated 129/Sv.

TABLE 1.—Incidences of teratomas in different sublines of strain 129

Colony	Number ♂♂	Percent teratomas
129/Re	382	0. 26
129/RrSv(ovary transplant animals)	5525	0. 45
129/Rr	1452	0. 55
129/RrSv	1067	0. 75
129/Dr	832	1. 09
129/Sv	7063	1. 58
129/J	588	1. 70

Substitution of genes C, P, *and* Sl.—As a result of examination of animals of early backcross generations, it became apparent that the incidence of teratomas was higher in known-Steel animals than in their recognized non-Steel and albino or pink-eyed litter mates. The colony was expanded, and the data presented in table 2 reveal that the tumor incidence is approximately twice as high in the former as in the latter classes of animals.

TABLE 2.—Incidence of teratomas according to coat color of 129/Sv-*Sl*

	Number ♂♂	Number of teratomas	Percent teratomas		
C-P-Sl	1011	70	6. 92		
C-P-slsl	1378	34	2. 47	*P*0.7	*P*0. 001
cc or *pp*	478	14	2. 87		
Total	2876	118	4. 10		

Nongenetic Influences on Incidence of Teratomas

Laterality influence.—It has been reported (*1, 14*) that the testicular teratomas of strain 129 mice occur more frequently in the left than in the right gonad. To date, 597 teratomas have been observed: 71 percent in the left testis, 27 in the right, and 2 percent bilaterally.

Influence of litter seriation and maternal age.—The teratoma incidence in first litters was compared with that in second and later litters. Data presented in table 3 show that tumors are approximately twice as frequent in second and later litters as in the first, and that this is true not only for the 129/Sv colony, but for all classes of offspring in the 129/Sv-*Sl* colony.

TABLE 3.—Incidence of teratomas according to litter number 129/Sv and 129/Sv-*Sl*

Colony	Litter number	Number ♂♂	Number of teratomas	Percent teratomas	*P*
129/Sv	1	1181	10	0. 85	
	2+	5882	102	1. 73	0. 05–0. 02
129/Sv-*Sl* *Sl/sl*	1	318	15	4. 72	
	2+	374	35	9. 36	0. 05–0. 02
129/Sv-*Sl* *sl/sl*	1	453	5	1. 10	
	2+	547	20	3. 66	0. 02
129/Sv-*Sl* *cc, pp*	1	148	2	1. 35	
	2+	181	7	3. 87	*
Total (129/Sv-*Sl*)	1	919	22	2. 39	
	2+	1102	62	5. 63	0. 001

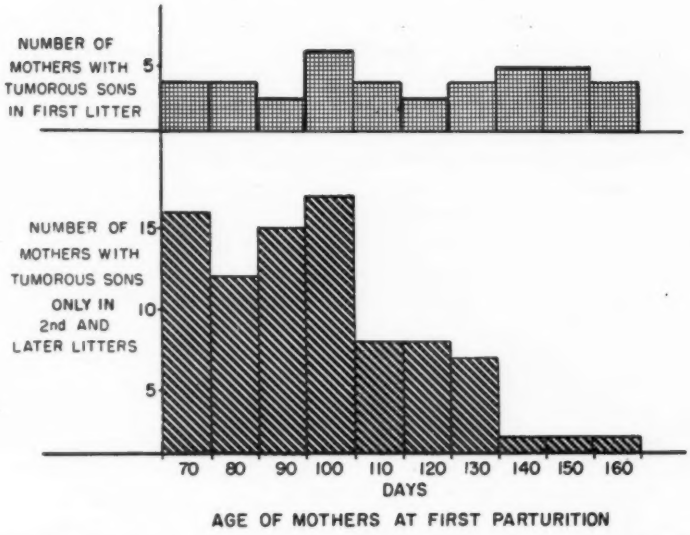
*A test for significance was not performed for this group.

The mean age of 32 mothers with affected sons in the first litter was greater at their first parturition than that of 79 mothers having affected sons only in the second and later litters. According to the result of a *t* test, the difference between the mean ages of the 2 groups of mothers is not significant ($0.10 > P > 0.05$). However, the distribution of the ages of mothers with tumorous sons in the first litter compared with that of mothers having tumorous sons only in second and later litters (text-fig. 1) suggests that at the time of first parturition, younger mothers may be less likely to have tumorous sons in their first litters than older mothers.

DISCUSSION

The relative contributions of tumor-susceptible genotypes and environmental factors to carcinogenesis have interested biologists for many years. Strains of genetically homogeneous animals, prone to develop particular types of tumors, have provided backgrounds against which both chromosomal and extrachromosomal influences have been investigated.

Subline differentiation accompanied by changes in mammary-tumor incidence is a well-known example of genetic influence on neoplastic


NUMBER OF MOTHERS WITH TUMOROUS SONS IN FIRST LITTER
5
NUMBER OF MOTHERS WITH TUMOROUS SONS ONLY IN 2nd AND LATER LITTERS
15
10
5
70 80 90 100 110 120 130 140 150 160
DAYS
AGE OF MOTHERS AT FIRST PARTURITION

TEXT-FIGURE 1.—Age distribution at first parturition of mothers having sons with teratomas in first litter compared with mothers having sons with teratomas only in second and later litters.

growth (*2*). Carpenter, Grüneberg, and Russell (*5*) investigated genetic differentiation involving morphological characteristics in sublines of the C57BL strain, and concluded that differences are "probably largely or entirely due to mutations which have happened since the sublines were separated from each other."

Testicular teratomas occur in a predictable proportion of strain 129 mice, and are almost unknown in other inbred strains, which indicates a genetic basis for their development. This concept is confirmed by the observation that there are appreciable differences in incidence between sublines of this strain. All sublines mentioned here descended from offspring of 1948 matings of a single male to several unrelated female inbred strain 129 mice. Brother × sister inbreeding was interrupted in 1947 when this laboratory was destroyed by fire. Some residual heterozygosity probably existed at that time.

Single genes have been shown to exert pronounced effects upon mammary and pulmonary carcinogenesis (*6*, *7*, *15*, *16*). An association between the gene yellow (A^y) and decreased mammary-tumor incidence but lowering of tumor age in mice derived from a cross between a strain high and a strain low in mammary-tumor incidence was demonstrated by Little (*6*). He suggested that the lower incidence of mammary tumors in yellows as compared with nonyellows might reflect a difference in hormonal stimulation of mammary tissue.

Such seemingly irrelevant genes as hairless, vestigial-tail, shaker-2, waved-2, fused, and flexed-tail reduce body weight and decrease the inci-

dence of lung tumors in inbred mice. Yet lethal yellow (A^y) increases both body weight and occurrence of pulmonary tumors (*17*).

Law (*8*) demonstrated an association between susceptibility to leukemia and the flexed-tail gene in mice. Heston (*17*) suggested that since flexed-tail influences the development of blood cells, its effect on the occurrence of leukemia may be through an early effect on the hematopoietic system.

Russell and Fekete (*9*) presented an interesting analysis of the effect of the gene Viable dominant spotting (W^v) on ovarian tumorigenesis. The ovaries of C57BL-W^vW^v mice contain very few germ cells and developing ovarian follicles which might be associated with a very low level of circulating estrogen. A low level of circulating estrogen promotes ovarian-tumor formation in mice, and tumors were observed in all W^vW^v females examined.

We have introduced several mutant genes onto the strain 129 background to determine what influence they would exert on the incidence of teratomas. Only Steel (*Sl*) has appreciably increased the incidence of tumors (from 2.5 to 6.9% in the 129/Sv-*Sl* subline). The gene Steel was selected because when homozygous it has a striking effect on the development of primordial germ cells. Bennett (*10*) reported a total lack of germ cells in *SlSl* embryos at 14 days' gestation, and the gonads of both sexes are henceforth devoid of germinal elements. In heterozygous (*Slsl*) animals, the germ cells are reduced in number in prenatal animals, although adults have histologically normal testes and fertility is not diminished (*10*).

We introduced the genes *C* and *P* with *Sl* to distinguish animals of the *Slsl* genotype from their *slsl* litter mates. Known-Steel animals have over twice as many teratomas as their recognized non-Steel and albino or pink-eyed litter mates. The influence of the *Sl* gene upon the incidence of teratomas in strain 129 mice is a new pleiotropic effect to be added to its other effects on the development of germ, pigment, and red blood cells. The effects of the *Sl* gene on germ-cell development and on teratocarcinogenesis may or may not be independent of each other.

The incidences of teratomas in recognized non-Steel (*C-P-slsl*) and albino or pink-eyed animals of the 129/Sv-*Sl* colony was higher than the incidence of teratomas in the 129/Sv colony (tables 2 and 3), none of which carried the genes *C* or *P*. Since recognized non-Steel animals were coisogenic with 129/Sv animals except for *C*, *P*, and possibly linked genes, these gene differences may have been associated with the difference in tumor incidence. The presence of Steel was not detectable in albino or pink-eyed animals, and this gene could have been responsible for the increased incidence of teratomas in this group.

Heston (*17*) suggested that genes which influence the occurrence of pulmonary tumors and have an effect on the normal growth of the animal "might be expected to have their action influencing pulmonary tumors manifested through some general mechanism of the host." The average weights of 60-day-old known-Steel, recognized non-Steel, and 129/Sv

virgin females were 18.9, 19.4, and 18.5 g, respectively, which indicates that the Steel gene does not affect body weight.

Sl^{d}, an allele of *Sl*, is being introduced onto the 129 genetic background as are *W* and *W*^{v}, alleles at another locus, with pleiotropic effects similar to those of *Sl* (*18*). Homozygous *Sl*^{d} and *W*^{v} animals are viable, which will make it feasible to compare the incidences of teratomas in normal and germ-cell-free litter mates. Observations on the effects of these genes on teratocarcinogenesis may help determine whether the *Sl* gene or closely linked genes are involved. These observations may permit identification of the cell of origin of testicular teratomas in mice.

In addition to genetic factors, numerous environmental factors have been shown to influence tumorigenesis in mice. MacDowell *et al.* (*19*) and Law (*20*) have demonstrated a progressive decrease in the incidence of leukemia with advancing parturition age of a low-leukemic strain mother. This influence is contributed prenatally and through the mother's milk, but specific characteristics of the maternal resistance factor are not known. Strong (*13*) showed that a maternal principle, as measured by litter seriation, affects susceptibility to chemically induced fibrosarcoma in mice, but its nature is not yet determined. Bittner (*21*) reported a positive correlation between maternal age and incidence of spontaneous mammary tumors in the progeny. He suggested that the titer of mammary-tumor agent may be higher in older mothers. Wright (*11, 12*) found that age of parents is an important factor in occurrence of polydactyly and in amounts of light spotting in guinea pigs. He suggested that the effect of age of dam on the piebald pattern might be considered an immaturity rather than a senescence effect, but its nature has not been determined.

The proportion of strain 129/Sv-*Sl* first-litter males with teratomas is approximately half that in animals in later litters. The increase in incidence from the first (2.4%) to the second (5.6%) litter is abrupt, and suggests the possibility that the maternal environmental influence has to do with age or parity of the mother. The incidence of teratomas in third and later litters (5.8%) is similar to the incidence in second litters, which indicates that the maternal influence may be established after the first parturition. This is evidence that susceptibility to teratocarcinogenesis may be influenced by the age of the mother. The distributions of the ages of mothers having tumorous sons in first litters compared with ages at first parturition of mothers having tumorous sons in second and later litters (text-fig. 1) also suggest that teratocarcinogenesis in first-litter males may be influenced by the age of the mother.

There is some evidence that tumors may be unevenly distributed among paired organs. For example, the second pair of nipple regions of RIIIb mice are more prone to form hyperplastic nodules than any other pair (*22*), and the distribution of mammary tumors in mice increases anteriorly among the glands without the mammary-tumor agent (*23*). Ovarian teratomas of human beings occur with a greater frequency in the left ovary than the right, while teratomas of the human testes show a pre-

dominance of growths on the right side (*24*). Testicular teratomas in mice occur on the left side more frequently than on the right (*1, 14, 25*) in very young animals, which indicates that a local environmental influence may affect the initiation of teratocarcinogenesis.

There are other examples of influence of maternal age, and asymmetry of expression on genetically determined traits (*26–29*), and according to Hauschka and Brown (*29*), "All the mammalian phenotypes for which significant fluctuations with maternal age are on record have one feature in common: they are controlled by genetically complex multifactorial and modifying mechanisms. Asymmetry in expression and sex-differences in manifestation are, also, frequently involved." The information at hand does not suggest a theory of teratocarcinogenesis that would explain the initiation of teratomas in strain 129 mice. The genetic and non-genetic factors involved appear to be similar to those which determine the penetrance of non-neoplastic inherited characteristics.

Thus far, the results of only those observations which showed a correlation with teratoma incidence have been mentioned. In addition, observations yielding little information were made. A brief discussion follows of these essentially negative results and relevant work done by others.

Landauer (*30*) pointed out that the presence of genes with low penetrance is likely to enhance the efficacy with which phenocopies, corresponding to the effects of these genes, can be produced. The influence of genetic factors is apparent in the development of spontaneous tumors and is equally important in induced tumors (*17*). For example, BALB/c mice are far more susceptible to estrogen-induced interstitial-cell tumors of the testis than other strains (*31*). We have made several unsuccessful attempts to induce germ-cell tumors in strain 129 mice. Since teratomas arise in very young animals, females pregnant 9 to 19 days and newborn male mice were treated with a variety of hormones and other agents with the aim of inducing teratomas in these genetically susceptible animals. The hormones and other agents included testosterone, adrenalin, noradrenalin, pitocin, pitressin, estradiol, progesterone, cortisone, urethane, cadmium chloride, zinc chloride, carbon tetrachloride, chloroform, X irradiation, and cell-free extracts of AKR leukemia. At about 10 days of age, 1,655 males which had been subjected to treatment were examined for the presence of teratomas. Of these, 7 had teratomas; however, it was not apparent that any treatment influenced the incidence of these tumors. It should be emphasized that, although we failed to obtain an increase in incidence, we did not demonstrate that any agent used was ineffective in inducing teratomas. Salivary-gland tumors developed in mice receiving injections shortly after birth of AKR leukemia extract, but the incidence of testicular teratomas was not affected.

Guthrie (*32*) attempted to induce tumors in the rodent testis to provide material for studying the histogenesis of the common human types, especially the seminoma. Several types of tumors were observed, but only one animal showed possible premalignant change in the seminiferous tubule. The results Guthrie obtained by injection of zinc salts were

essentially negative in producing testicular malignancy. In view of the success others have had in this direction (*33*), we are currently injecting zinc salts into the testis of strain 129 mice which are genetically susceptible to tumors of probable germ-cell origin.

Little (*34*) crossed 2 species of mice and found that the incidence of tumors in the first-generation hybrids was greatly increased. Dickie (*35*) found that ovarian-tumor incidence in CE and DBA backcrosses gave an excellent example of a condition appearing to a greater extent in the backcross generation than in either of the parent strains or in the F_1 hybrids. The incidence of ovarian tumors in CE females was 34 percent, in DBA females, 0, in DBA-CE hybrid females 33, and in CE-DBA, 19 percent. CE backcrosses had an incidence of 52 percent, an enhanced incidence over the 34 percent available from strain CE. With the aim of finding a genetic background which would enhance the incidence of teratomas, strain 129 mice were reciprocally crossed to strains DBA, CE, BALB/c, TC3H, C57L, A/He, and AKR. Male and female F_1 hybrids from these crosses were backcrossed to strain 129 mice. From these crosses and backcrosses 11,292 males were examined for tumors, and 1 teratoma was found in a BALB/c backcross male. Apparently testicular teratomas in mice are determined by multiple factors present in the inbred strain 129, and attempts to enhance the incidence of tumors by the use of different genetic backgrounds failed.

Several specific genes other than Steel were backcrossed for at least 2 generations to the strain 129 genetic background for determination of their influence on the incidence of teratomas. *C* and *P* were introduced together, and diminutive (*dm*), Disorganization (*Ds*), and Fused (*Fu*) were introduced singly. Other mutations occurred in colonies of strain 129 mice and included dystrophia muscularis (*dy*), jaundiced (*ja*), open eyelids (*oe*), Rib fusions (*Rf*), and joined toes (*jt*). Exhaustive breeding tests were not performed, but it was apparent that none of these genes greatly increased the incidence of teratomas.

Spontaneous testicular teratomas are believed to arise early in development in human beings and horses (*36*) and are known to be present at birth in strain 129 male mice (*1*). The high incidence of spontaneous teratomas in the left testes of late-litter mice of the 129/Sv-*Sl* colony has made it feasible to study the development of these tumors by examining serial sections of 15- to 19-day fetal testes.

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"Safety" Testing of Carcinogenic Agents[1]

NATHAN MANTEL *and* W. RAY BRYAN, *Biometry Branch and Laboratory of Viral Oncology, National Cancer Institute,*[2] *Bethesda, Maryland*

SUMMARY

The problem of determining what dose levels of an agent are safe, *e.g.*, noncarcinogenic, cannot be resolved unless one first defines some level of permissible risk, no matter how small, rather than insisting on absolute safety. Both because of practical considerations and statistical variation, the determination of low-risk dose levels, for example 1/100 million, cannot be made directly but must be by extrapolation from observed data. A conservative approach for doing so is given. In addition to an arbitrary definition of "virtual safety," it is necessary to define an arbitrarily high statistical assurance level and a rule for extrapolation by use of an arbitrarily shallow slope. Illustrative data by Bryan and Shimkin (J. Nat. Cancer Inst. 3: 503–531, 1943) on the carcinogenic action of methylcholanthrene yield a "safe," 1/100 million, dose of 9×10^{-5} mg per mouse when a statistical assurance level of 99 percent and a conservative probit slope of 1 normal deviate per log for extrapolation are used. The principles given are of general applicability in other safety-testing problems, the point of emphasis being that since direct observation cannot be made that the risk at some dose level is clearly low, indirect conservative procedures for the determination of low risk levels must be made. The arbitrary risks and definitions for so doing may change with circumstances. The procedure does not require specification of an experimental protocol; the "safe" dose is determined on the basis of whatever data are available. Minimum protocols may, however, be desirable, since greater amounts of data will ordinarily permit specifying large "safe" levels.—J. Nat. Cancer Inst. 27: 455–470, 1961.

ALTHOUGH IT is not definitely known whether all chemical compounds that induce cancer in experimental animals will also cause cancer in man, it has been fairly well established that, with the possible exception of arsenical compounds, every chemical or physical agent known to produce cancer in man will likewise do so in one or more species of lower animals (*1*).

Of necessity, the potential deleterious effects of chemical compounds must be tested in laboratory animals. The reaction of a particular species of animal does not constitute proof that humans will react similarly, but

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the only recourse the investigator has in implementing test programs for control of the human environment with respect to injurious chemical or physical agents is to proceed under the assumption that any substance harmful to animals is potentially harmful to man. Also in carrying out control tests in animals one must proceed, initially, as if protection of the animal population were the actual problem. When reliable estimates of the doses tolerated (with specified probability) are achieved for the animal population, the problem then becomes one of judgment, based on accumulative experience, in transferring the implications of the results to man. Results of testing with a variety of animals could suggest how extrapolation could be made properly to mammalian species of higher order or larger size.

Many chemical compounds may be harmful at certain concentrations, though beneficial at others. The control of "toxic" or "harmful" substances therefore does not imply the necessity of their complete or absolute elimination, which in some cases would be either impossible or economically infeasible, but their reduction to concentrations that can be tolerated by essentially all individuals of the population at risk.

In rapidly acting toxic substances that are either quickly eliminated from the body or are readily transformed to less harmful compounds through metabolic processes, the estimation of tolerated levels is not too difficult. With a reasonable allowance for an extra margin of safety introduced in the form of an arbitrary "safety factor," the results obtained in laboratory animals can be successfully projected to humans. For the most part, modern pharmacology is based on just such usage of laboratory animals.

There are other compounds, however, for which the results obtained in laboratory animals cannot be so confidently projected to man. These substances are not readily excreted or metabolized, and because of their weak solubility in aqueous solution, may remain in the body or on body surfaces for very long periods. Other substances may be metabolized to some extent, but because of their selective affinity for cells of certain types they may accumulate selectively within these cells to yield harmfully high concentrations if supplied on a continuing basis. Most chemical compounds that cause cancer, *e.g.*, polycyclic aromatic hydrocarbons, naphthylamines, azo dyes, and some steroids, have properties falling into one or the other of these categories.

Still other compounds have an immediate initial effect, which is not considered harmful, and are rapidly eliminated or metabolized; yet, during their brief sojourn in the body they produce some critical intracellular damage or change, which does not manifest itself until later. Urethan, which interferes with nucleic acid metabolism, is a classical example of a compound of this type. Injected parenterally, urethan acts immediately to induce transient anesthesia, but single anesthetic doses may cause lung tumors in mice many months later. Fortunately, this drug was never approved for use as an anesthetic for man.

Biometric methodology has been highly developed for the study of

acute toxicity and rapidly developing biological reactions of other types (*see 2–4* for review). Some progress has been made in the development of methods for the analysis of responses that are greatly prolonged in time, such as cancer (*see 5* for review); however, the problems associated with the estimation of tolerated levels of carcinogenic agents and other chronically acting compounds have not yet received adequate study.

The purpose of this communication is to discuss the major problems in the development of methods for estimating limits of tolerance, or "safety levels," of carcinogenic compounds and to describe certain biometric procedures, based on available data, that are applicable to presently known carcinogenic compounds and experimental animal systems. Factors which one must consider in transferring the inferences derived from results in animals to man are also discussed. The suggested procedures are not restricted to carcinogenic agents, but are applicable, in principle, to compounds which cause various other types of harmful reactions or disease.

SOME PROBLEMS IN PLANNING AND ANALYSIS OF SAFETY STUDIES

In a safety-testing program both the design of the experimental protocols and the method of analysis or interpretation of resulting data must be determined. The experimental design cannot be properly determined apart from the plan for analysis of the data.

Certain issues in a safety-testing program must be resolved in advance. These relate to what we mean by safety and what kind of feasible results we are willing to accept as proof of safety. Settling of these issues, if necessary on some arbitrary but conservative basis, may permit answers to problems that would otherwise be insoluble. These problems are:

1) *How safe is safe?* Absolute safety can never be unquestionably demonstrated experimentally. Rather, experimental results can be used only to establish limits on the risk involved. With the specification of some level of risk, no matter how small, the possibility of determining whether or not that risk is exceeded opens. We may, for example, assume that a risk of 1/100 million is so low as to constitute "virtual safety." Other arbitrary definitions of "virtual safety" may be employed as conditions require.

Incidentally, an inflexible requirement for absolute safety may lead to acceptance of high levels of hazard. The impossibility of really demonstrating absolute safety leads to the acceptance, as a satisfactory demonstration, that no hazard was observed in an experimental protocol of moderately large size, 100 or even 1,000 animals. Such evidence, however, only provides assurance, at the 99 percent probability level, that the true risk is under 4.5 percent in the 100 animals or 0.46 percent in the 1,000 animals.

2) *What constitutes proof of safety?* In principle, one could use an experimental protocol sufficiently large to demonstrate that "virtual

safety" obtained. For this purpose it must be realized that an observed outcome of no tumors among 100 million treated mice does not necessarily demonstrate clearly that treatment was either absolutely or even virtually safe. This outcome could arise with a probability of 1 percent, even if the risk involved were as high as 4.6/100 million. It would in fact require a total of some 460 million tumor-free mice to demonstrate at the 99 percent assurance level that "virtual safety" obtained. Similarly, tumor-free results for 10,000 mice would only indicate that the risk was less than 1/2,200 and it would require tumor-free results in a total of some 450 mice to establish with high probability that the risk was under 1 percent. Studies of feasible size can be used to establish directly only risks of the order of 1/100 or higher. Data from such studies can be used to ascertain the treatment level consonant with a prescribed risk or to establish limits on the risk for a particular treatment level. The determination of "safe" levels can be made only by indirect methods extrapolating from the data obtained in a feasible study.

The use of extremely large studies to establish safety may well be self-defeating. The almost certain occurrence of unusual syndromes in one or more of a large number of test animals, albeit these may have arisen spontaneously, will require admitting the possibility that they may be attributable to drug treatment.

3) *How can protocol data be extrapolated safely?* Since it is only feasible to use experimental protocols for the direct determination of relatively high-risk dose levels, *e.g.*, 1 percent, which makes extrapolation methods necessary, one must consider that such extrapolation methods might yield misleading results. Procedures exist which permit extrapolating the results obtained at a number of test-agent levels to determine the dose level corresponding to any desired degree of risk and to establish, with a high level of assurance, a minimum bound on this dose level. These methods, however, are based on the assumption that the relationship observed between tumor occurrence and dose at the levels tested will continue to apply in the regions to which extrapolation is being made. The validity of such an assumption cannot be tested and, if it is false, may lead to a serious overestimate of the "safe" level. Such overestimation would arise if the relationship of response to dose is less pronounced at the dose levels to which extrapolation is made than at the levels at which tests were performed. Another related source of difficulty is that tests are performed on relatively pure inbred strains of laboratory animals. Characteristically, such pure strains will show steep dose-response relationships, while the heterogeneous population to which it is intended to apply the results of testing may exhibit a shallow relationship.

To avoid the risk of overestimation of "safe" levels which may result from extrapolating with too steep a slope, it is suggested here that a conservative result may be obtained by extrapolation with an arbitrarily low slope from the data at hand. For example, quantal-response data, that is, all-or-none response, frequently exhibit a somewhat linear relationship when plotted on probability paper with a normal or probit

scaling for the percent responding and a logarithmic scaling on dose. According to the kind of system being investigated the dose-response slopes observed may vary widely. With systemic poisons, rather steep dose-response slopes are generally obtained, reflecting the narrow logarithmic range between the lowest dose levels at which any toxic deaths occur and the levels which are lethal for all animals—such response slopes are on the order of 10 to 50 or more probits per common logarithmic or tenfold dose increase (the technical definition of this slope is not given here; it permits one to perform any necessary extrapolations). The all-or-none response also arises in the study of the therapeutic effects of antibiotics. The response slopes in these instances are generally shallower, on the order of 3 probits per common logarithm. Lower slopes on the order of 2 do arise in virus-assay work, but in these instances it may be that use of a different response curve, the single-hit or one-particle curve, would be more appropriate. From such experience it would appear that the use for purposes of extrapolation of a slope as low as one probit per common logarithm is likely to be conservative. While slopes in the regions to which we wish to extrapolate cannot be established, the suggested slope of one is rather low compared with that ordinarily obtained in the observable region.

The indicated low slope is a key feature in the method to be suggested as conservative for the establishment of "safe" levels. For this reason it may be well to make clear just how weak or strong are the assumptions being made in its use. In fact the only assumption being made for the procedure to be conservative is that whatever form the true response curve may take over the region of extrapolation, the average slope is not less than the assumed one. There is no requirement that the true response curve be linear or even that the true slope should nowhere be less than assumed. The use of the indicated conservative slope is, of course, arbitrary. Other values may be specified and other scales for extrapolation may be employed.

Once answers to the three questions are provided, a defined level of "virtual safety," a prescribed level of statistical assurance, and a conservative rule for extrapolation, it becomes possible to determine, from protocol data, "safe" dose levels and to undertake the planning of any necessary experimental protocols. This is considered in the succeeding sections.

ANALYSIS OF RESULTS AT A SINGLE DOSE LEVEL

In what follows we will take the defined level of "virtual safety" to be 1/100 million, the statistical assurance level to be 99 percent. Extrapolation will be on the basis of 1 normal deviate or probit per common log or tenfold change in dose.

To illustrate how definitive "safe" levels are obtained, consider that a prescribed dose of an agent has elicited no tumors in a group of 100 ex-

perimental animals. While the observed rate of tumor occurrence is 0 percent, we will take, as an upper limit on the true rate, that risk for which the probability for occurrence of as few as zero tumors is 1 percent (100% less the assurance level of 99%). This is given by the solution for P to the equation

$$(1 - P)^{100} = 0.01.$$

Solving, we have

$$100 \log (1 - P) = \log 0.01 = -2; \log (1 - P) = -0.02 = 9.98\text{–}10;$$
$$1 - P = 0.955; P = 0.045 \text{ or } 4.5 \text{ percent.}$$

We now know that the observed outcome of no tumors in 100 animals is consistent with the possibility that the true risk was, in fact, 4.5 percent. From tables of the normal probability function (*6*) we determine that the normal deviate, Y, such that the integral from $-\infty$ to Y equals 0.045 is -1.695, for a probit value of $3.305 = 5 - 1.695$. However, the normal deviate, Y_e, corresponding to a risk of 1/100 million can similarly be determined as -5.612, the probit being -0.612. The upper limit on the risk for the dose employed is $3.917 = -1.695 - (-5.612)$ normal deviates above the desired safe risk, and, at a slope of one normal deviate per common log, it is necessary to reduce the log dose by 3.917 logs to attain a "safe" level. The antilog of 3.917 being about 8,300, it is determined that the "safe" dose is 1/8,300 times that which had been tested.

Table 1 shows the preceding results together with those for several other hypothetical experiment sizes in which no tumors were observed to occur in a group of treated mice. For each such group the table shows the greatest risk consistent, at the 99 percent assurance level, with the observed outcome. Also shown are the corresponding conservative estimates, with the slope of one normal deviate per tenfold dose increase, of the "safe" (1/100 million) dose level expressed as a fraction of the dosage tested. The larger the experimental group among which no tumors occurred, the greater is the value determined as the "safe" dose.

TABLE 1.—Illustration of "safe" doses determined when no risk was observed in single groups

Number of mice with tumors/ No. tested	0/10	0/50	0/100	0/500	0/1,000
Upper limit on tumor risk at level employed, 99 percent assurance (percent)	37	8.8	4.5	0.92	0.45
Estimated "safe" dose (1/100 million) dose employed = 1	1/190,000	1/18,000	1/8,300	1/1,800	1/1,000

Study of this table indicates that a control system can be established without the need for specifying a design protocol, though there might be some merit in specifying a minimum size. For, whether the amount of evidence adduced to show that an agent is safe is great or small, it can be properly weighted to determine conservative safe limits. If the promulgator of a drug wishes to have high tolerances established for his

compound, it would be worth while for him to produce results of experiments with a large number of animals, which would show that the agent is not especially dangerous at certain dose levels. Where the lack of danger is demonstrated with a small number of animals or as a result of testing at rather low doses, the dose levels determined as "safe" will be lower. A control system can be constructed about the possibility for interpreting the data submitted, with no specification needed as to how much data should be obtained.

THE CASE OF SOME OBSERVED RISK

The method indicated for determining "safe" levels is not restricted to the case in which no observable danger was noted. That case was used for illustration because of the simpler mathematical solution involved. In general it will be that an experiment testing n animals will yield r unfavorable results (tumors). The upper limit on risk at the 99 percent assurance level is then the solution for P to

$$\sum_{i=0}^{r} {}_nC_i P^i (1-P)^{n-i} = 0.01$$

or

$$\sum_{i=r+1}^{n} {}_nC_i P^i (1-P)^{n-i} = 0.99$$

The solution for P is a value such that the chance of observing as few or fewer than r tumors is 1 percent. At values for P in excess of the solution, the chance for such an outcome is less than 1 percent.

The preceding equation can be solved approximately by reference to tables of cumulative binomial probabilities. Such tables, as for examples those of the Ordnance Corps (7), show values of quantities such as

$$\sum_{i=r+1}^{n} {}_nC_i P^i (1-P)^{n-i}.$$

Let us consider as a hypothetical outcome that, of $n = 100$ mice, $r = 10$ have developed tumors, for an observed rate of 10 percent. Referring to these tables we see that for $P = 0.19$

$$\sum_{i=11}^{100} {}_nC_i P^i (1-P)^{n-i} = 0.9891$$

and that for $P = 0.20$

$$\sum_{i=11}^{100} {}_nC_i P^i (1-P)^{n-i} = 0.9943.$$

We may take the solution for P as approximately 0.192. The calculating procedure follows as before. The normal deviate corresponding to a 19.2 percent probability is -0.871 and, at the slope assumed, it will require a reduction in log dose of $4.741 = -0.871-(-5.612)$ to obtain a risk of 1/100 million. The "safe" dose is then determined as 1/55,000 of the dose which had been employed, 55,000 being the antilog of 4.741.

USE OF CONTROL DATA

In the methodology shown, it was assumed that the response of interest, appearance of tumors, did not occur spontaneously. In general, however, it will be desirable to use controls to check this and to allow data obtained for such controls to modify the determination of "safe" dose made. However, with the method already shown, failure to use controls or to take control data into account will result in more conservative determinations of the "safe" dose. If, in fact, spontaneous rates are rather low, they will have little effect on the determination made. For this reason we may adopt a procedure which is somewhat more conservative than necessary for taking control data into account. This we can do by taking, as before, the upper limit for the risk in the treated group as the solution for P_t to

$$\sum_{i=0}^{r_t} n_t C_i P_t^i (1 - P_t)^{n_t - i} = 0.01$$

while the lower limit on the control group risk is the solution for P_c to

$$\sum_{i=r_c}^{n_c} n_c C_i P_c^i (1 - P_c)^{n_c - i} = 0.01$$

where r_t of n_t treated animals and r_c of n_c control animals, respectively, showed positive response. These equations can be solved through the use of binomial tables.

At this point Abbott's formula (*8*) can be used to obtain a modified value for the treated-group risk, this being computed as

$$P_t' = (P_t - P_c)/ (1-P_c).$$

The computation follows as before, the normal deviate being obtained corresponding to P_t'.[3] Since P_t' cannot exceed P_t, the use of control data cannot result in decreased values for the calculated "safe" dose.

ANALYSIS OF RESULTS OBTAINED AT SEVERAL DOSE LEVELS

When an agent is tested, it is sometimes desirable to do so over a number, perhaps even a wide range, of dose levels. In such instance, all the available data should be considered in the determination of "safe" levels.

[3] The fastidious statistician may object to the moderately conservative procedure described here for determining P'_t. A more rigorous procedure would require setting limits on the ratio of binomial parameters. P_t' would be given by reducing by unity the upper limit on the ratio of control-to-treatment nonresponse probabilities.

Parametric Procedures

As already indicated, it may be unwise to extrapolate the data with the observed response slope. Procedures for taking into account the statistical variation of the fitted slope will not suffice to make such extrapolation methods conservative. To see this, one need only consider the use of quite large study sizes. In this case, statistical variation will be negligible with the result that extrapolation to low-risk levels will be substantially with the slope obtaining in the observable range. (However, with small study sizes or studies, the designs of which are inefficient for estimation of the slope, taking account of the statistical variation of the slope determination may result in extremely conservative estimates of "safe" levels. The lower confidence limit on the "safe" dose in such instances may be less than that which would be obtained when an arbitrarily low slope value is used for extrapolation, as suggested.)

An alternative device could be to employ parametric procedures, for example, fitting the maximum likelihood probit line to the data (*9*), to determine the lower confidence limit on the dose corresponding to some moderate percentage risk, *e.g.*, 1 percent, to which risk-level extrapolation with the observed slope is considered reliable. Then, using an arbitrarily conservative value for the slope, and anchoring at the lower limit on the 1 percent dosage, one could extrapolate to the desired "safe" level.

While the procedure just indicated is straightforward, its employment is based on the validity of the parametric function employed. Quite useful results have derived from such parametric assumptions in bioassay work. But for bioassay purposes it is not essential that a parametric function be exactly appropriate; it is sufficient that the function assumed do a reasonably good job of graduation. (Even somewhat inappropriate curve forms can yield reasonably good relative potency estimates as long as the preparations being compared are tested over the same regions of response.) The use of an invalid parametric function can lead to inappropriate estimates of dose levels corresponding even to moderate risks.

In many situations the estimates may be only moderately inappropriate, in others quite serious. With parametric procedures, the use of rather large experimental groups in one region of the response curve can be reflected in narrow-range confidence intervals for dose levels corresponding to risks in other ranges. This can produce a false sense of security in one's estimate of the moderate-risk dose level when the parametric model is violated.

Accordingly, while agreeing that parametric procedures may be useful, we will consider the possibility for extending the method described for the single dose-level case without the need for assuming any particular model. The only assumption is that the arbitrary low slope assumed is conservative and it is, of course, implicit that the response curve is monotone. In instances in which it can be recognized that use of a parametric procedure is not misleading, workers may prefer this procedure rather than the more generally appropriate nonparametric procedures described in the next section.

Nonparametric Procedures

In the preceding section it was suggested that the use of parametric methods, while straightforward, could lead to nonconservative results. There are no simple fixed rules for conservative estimates of the "safe" dose when several dose levels are employed and one is unwilling to make assumptions about the dose-response curve in the region of observation. How estimates can be made in these circumstances can best be demonstrated by illustration.[4]

We will begin with some simple ideas. Suppose investigators at two laboratories independently test an agent at a level of 100 mg/kg. At the first, with 500 mice tested, no tumors are observed, and with the methods described previously the "safe" dose is estimated as 100 mg/kg/1,800 = 0.056 mg/kg (*cf.* table 1). A somewhat lower "safe" dose of 0.012 mg/kg is obtained at the second laboratory, based on the observation of no tumors among only 100 mice. It can readily be recognized here that it would be inappropriate to reject the high "safe" level of the first laboratory just because of the low estimate obtained at the second laboratory. The two sets of data are consistent and in fact confirm each other. If any modification is to be made, it should be to consider that, with results combined, no tumors have occurred among 600 mice which would lead to a safe dose of about 0.065 mg/kg.

Suppose that at still a third laboratory, tests are made at a dose of 50 mg/kg and, with no tumors occurring among 500 mice, the calculated "safe" dose at that laboratory is 0.028 mg/kg. Here again we can see that the "safe" dose obtained at the first laboratory should not be modified downward just because a consistent result at the third laboratory yielded a lower "safe" dose. If anything, it should be considered that the 500 mice not responding at the higher dose at laboratory 1 would not have responded at the lower dose employed at laboratory 3. With these 500 mice treated as nonresponders at the low dose, there is then, including those at laboratory 3, a total of 1,000 mice not responding at the low dose. (Laboratory 2 results are being ignored for this illustration.) This yields as a calculated "safe" dose 50 mg/kg/1,000 = 0.050 mg/kg. In the present case the "safe" dose based on the combined calculation is less than that for the data of laboratory 1 alone of 0.056 mg/kg and so the higher figure is retained. Had the combined calculation led to a higher "safe" dose it would have been correct to take that as the estimate.

The point of these illustrations is that, when the data obtained from a series of doses are consistent with each other, it is appropriate to take as the calculated "safe" level the highest one pertaining to the results at any one dose. Even a higher "safe" level may be taken when it can be obtained through a justifiable combination of the results at the various doses used. What is meant here by "justifiable" combinations can be seen from the following example in which hypothetical results at four dose levels, low, middle, and high, are considered.

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[4] An alternative method to the one about to be described is given as an appendix.

Dose in size order	Observed results Number of tumors/ number of mice	"Justifiable" combined results Combined number of tumors/combined number of mice			
1	0/100	(0/100);[1]	0/200;	1/300;	5/400
2	0/100	0/100;	1/200;	5/300	
3	1/100	1/100;	5/200		
4	4/100	4/100			

[1] Parentheses indicate that this result need not be considered, as the next must yield a higher value for the "safe" dose.

In the absence of inversions in the data, we can determine the "justifiable" combined results at a dose by adding to the results at that dose, in succession, the results at still higher doses. A calculated "safe" dose can be determined for each dose used and for each of the various "justifiable" combined results corresponding to each dose. The over-all "safe" dose would be the highest of the various determinations.

Where data show an inversion the procedure is altered. Consider a simple example:

Dose in size order	Observed results Number of tumors/ number of mice	"Justifiable" combined results Combined No. of tumors/combined No. of mice
1	1/100	(1/100);1/200
2	0/100	[(0/100);1/100]

At the first dose level it is clear that the calculated "safe" dose would be larger if based on the combined results for both levels than if based on the results observed at this level alone; accordingly, as noted in one instance in the preceding example, the result at the lower level alone is shown in parentheses. At the higher dose level an inversion occurs; there is a lower incidence of tumors even though the dose level is higher. In view of the inversion, one would be less willing to accept as "safe" the calculated value obtained on the basis of results for this dose level alone. The two alternative results shown in brackets at this dose level are the results at this dose level and the contradictory results at the lower dose level. The significance of the use of brackets here is that the calculated "safe" value is now to be taken as the lesser of the values suggested by the alternative results. In the present instance, the result at the lower dose 1/100 would yield the lower "safe" dose and so the alternative result is shown in parentheses. (It will not always be necessarily true, when an inversion occurs, that the retained result will correspond to the higher tumor incidence at the lower dose level.) In the present example the calculated "safe" dose will be that corresponding to the first dose with combined result 1/200 or that corresponding to the second dose with retained result 1/100, whichever is the greater.

In practice the application of the methods just indicated is much simpler than the explanation would suggest. Ordinarily only one or perhaps two of the combined results at a dose level will need to be considered.

The results at some dose levels may immediately permit us to drop them from consideration. After only a limited amount of experience it should be possible so do this rather rapidly. The calculations are actually simpler than those for the maximum likelihood probit method. [In fact, the confidence limit procedures ordinarily employed in connection with the probit method are not fully satisfactory. A more appropriate method is described by Mantel and Patwary (*10*), but it could require a somewhat extravagant level of computational effort.] And, while for completeness, we have indicated the need for considering the possibility of inversions, this will ordinarily not pose a problem.

An Illustrative Example

An example from the literature shows how the procedure just discussed can be applied. The data, from Bryan and Shimkin (*11*), are the results obtained after a single injection of methylcholanthrene into mice, 12 different dose levels being used in the study. The reader may refer to the original article for details.

No peculiarities arise in this example. There are no inversions. At the four lowest levels no tumors occurred and the appropriate combined result is readily recognized in these instances. At the middle four levels it can be recognized that there is no point in combining results and, finally, the four highest levels can be disregarded as these all yielded 100 percent tumor occurrence.

The procedure is illustrated in table 2. The first three columns show, respectively, the dose, log dose, and the observed result. Column 4 shows each combined result considered, and there should be a separate line for each such result. In the present instance only one combined result required to be considered at each dose. For each such result, column 5 shows the calculated maximum risk at the 99 percent assurance level. These were obtained from binomial tables or calculated directly for the case of no tumors occurring. The normal deviate corresponding to the maximum risk is obtained from tables of the normal distribution and is shown in column 6. Finally, column 7 shows the calculated "safe" (1/100 million) log dose. The maximum for this, 2.962–10, appears in the second line, and the over-all calculated "safe" dose is 9×10^{-3} mg per mouse.

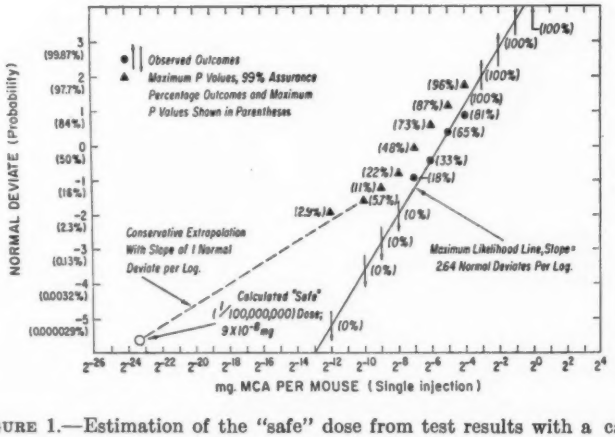
One might remark that this "safe" dose is so low as to make impractical any use of it which may result in its ingestion by humans. But we are dealing here with a rather potent carcinogen and if any compounds are to be assigned tolerated levels which are virtually zero, this is one of them.

Text-figure 1 shows graphically the results and analysis of the experiment just considered. Normal deviates are shown on the vertical scale, while on the horizontal scale the dose employed is shown as negative descending powers of 2. The points shown represent the outcomes at each dose level; 0 and 100 percent outcomes are shown by arrow. The solid line on the figure is the maximum likelihood probit line fitted to the

TABLE 2.—Illustration of methodology for determining the "safe" dose from results at several dose levels; data from Bryan and Shimkin (*11*)

Dose mg/mouse	Log dose	Result: No. of tumors / No. of mice	Combined result: No. of tumors / No. of mice	Maximum *P* value 99% assurance	Corresponding normal deviate	Calculated "safe" (1/100 million) log dose (2)−(6) −5.612
(1)	(2)	(3)	(4)	(5)	(6)	(7)
0.000244	6.388–10	0/79	0/158	0.0288	−1.899	2.675–10
0.000975	6.990–10	0/41	0/79	0.0566	−1.584	*2.962–10*
0.00195	7.291–10	0/19	0/38	0.1141	−1.205	2.884–10
0.0039	7.592–10	0/19	0/19	0.2152	−0.789	2.769–10
0.0078	7.893–10	3/17	3/17	0.480	−0.050	2.331–10
0.0156	8.194–10	6/18	6/18	0.729	+0.610	1.972–10
0.0312	8.495–10	13/20	13/20	0.871	+1.131	1.752–10
0.0625	8.796–10	17/21	17/21	0.958	+1.728	1.456–10
0.125	9.097–10	21/21	—	—	—	—
0.25	9.398–10	21/21	—	—	—	—
0.50	9.699–10	21/21	—	—	—	—
1.0	10.000–10	20/20	—	—	—	—

data. Above the first 8 data points the triangles shown correspond to the maximum *P* values of table 1. Extrapolation, with the slope of one normal deviate per common log to the over-all calculated "safe" value, is indicated by a broken line. All triangles, other than the one from which



TEXT-FIGURE 1.—Estimation of the "safe" dose from test results with a carcinogen methylcholanthrene, at several dose levels. At each test level both the observed percentage response and an upper limit, 99 percent assurance, based on combined data are shown. *Solid line* is the maximum likelihood probit line fitted to the data. The "safe" level of 9×10^{-8} mg per mouse is in this instance estimated by extrapolation with the conservative slope of 1 normal deviate per log from the upper limit on *P* at the second dose level.

extrapolation was made, should fall above the line. The difference in slope between the solid and the broken line may be noted.

RELATION TO OTHER SYSTEMS

While at various points in the preceding section the possibility for arbitrary selection of the assurance level, the level of safety desired, the slope value used for extrapolation, and even the extrapolation curve (one could use some scale other than normal deviates or probits along which a risk probability may be defined) have been emphasized, this should not be taken to mean that the methods suggested are completely general. Rather, in each case the nature of the risk situation should be considered.

Some principles do carry over, however. For example, one may be interested in trying to extinguish a bacterial or viral population by exposure to increasing temperatures or by increasingly long exposure to bactericidal or viricidal conditions. Determining the appropriate temperature or duration of exposure through the use of a conservatively shallow slope may be appropriate in such cases.

Consideration of the "single-particle" or "one-hit" problem (*12*) gives rise to a quite different answer than that developed in the preceding section. In this problem it is considered that a single particle can cause infection or death. If particles are distributed at random in material being inoculated, so that if there is an average of m particles per inoculation, the probability that a particular inoculation will contain none and so be safe is e^{-m}; the probability that it will not be safe is then $1-e^{-m}$. The problem is to determine from test data what reduction in the inoculum is necessary to ensure safety.

One can use directly the methodology already given to set maximum values on the risk as a result of the outcome of testing. This in turn establishes a maximum value for m as $-\log_e$(1-max. P value), the necessary reduction to any desired risk level following directly.

In this instance the inoculum corresponding to a risk of 1/100 million is approximately one millionth that corresponding to a risk of 1 percent. This contrasts sharply with the ratio of about 1/2,000 when extrapolation is made between these two risk levels with a slope of one probit per common log. While it has been shown that in the central region the "one-particle" curve mimics the probit curve with a slope of 2 probits per common log (*13*), the contrast would suggest that the comparatively steep slope of 2 noted virtually disappears in the lower tail. What further suggests itself is that the "single-particle" curve provides a most conservative rule for extrapolation. Where there is any suspicion that this curve may apply, the procedure described in the text should not be used.

One might also visualize a two-hit or two-stage process, the low-level probabilities for each stage being approximately proportional to the dose used. A reduction in dose by a factor of 1,000 should reduce the joint probability by a factor of 1 million. In this case the 1/100 million risk dosage would be 1/1,000th that of the 1 percent risk dose.

APPENDIX

Alternative Method

An alternative method to that described for handling data at several dose levels may be more appealing to the biometrically oriented reader. It was evolved in discussions with a reviewer (J. Cornfield) and is objective and intuitively satisfying.

In this method the data are considered to consist of a series of assays: one comprising results at the lowest dose level only; another comprising results at the two lowest levels; still another in which the results are given for those at the three lowest levels, etc. (Levels where there is interference, as for example lethality interfering with a carcinogenic response, should not be included.) For each assay, with appropriate probit procedures determine the maximum likelihood, 1/100 million dose level and its lower limit, subject to the restriction that the probit slope is known to be unity. Of all the lower limits on the 1/100 million dose level determined for the series of assays, the largest is selected as the "safe" dose.

A drawback to this procedure could be that standard methodology does not correctly permit the determination of lower limits on the 1/100 million level. Such methodology goes awry if, for example, there are no positive responses at the three lowest dose levels. A principle described by Mantel and Patwary (*10*) permits a solution. Calculate the chi-square goodness-of-fit, χ_1^2, (or log likelihood for those so inclined) for the maximum likelihood solution. (This chi square would be taken as zero if there are no positive responses at any of the dose levels considered; the maximum likelihood 1/100 million level is actually infinite. A zero chi square obtains also when only results at the lowest dose level are considered.) Consider alternative trial values for the 1/100 million dose level less than the maximum likelihood estimate. Each such trial value, in conjunction with the assumed slope of unity, provides an estimate of the response rate at each dose level of the assay. There is thus, in turn, a chi-square value, χ_2^2, for departures of the observed responses from the estimated responses based on the trial 1/100 million dose level. χ_2^2 will exceed χ_1^2 and for some trial value $\chi_2^2 - \chi_1^2$ will equal 5.412, the 98 percent upper limit on a chi square with one degree of freedom (a 98% limit on chi square corresponds to a 99% on the "safe" level).

At this point we introduce a modification which, for simplicity, has just been glossed over. Instead of computing χ_1^2 based on weights implied by the maximum likelihood solution, one should compute the quantity χ_1^{2*} based on departures from the maximum likelihood fit, the weightings being derived from the fit to the trial "safe" dose.

To illustrate: Suppose that at the i'th dose level, r_i of n_i animals respond; that the maximum likelihood estimate of the response rate is $\hat{P}_i$ and the estimate corresponding to the trial value considered is $\hat{P}_i^*$. Then

$$\chi_2^2 = \sum_i (r_i - n_i\hat{P}_i^*)^2/n_i\hat{P}_i^*(1 - \hat{P}_i^*)$$

and

$$\chi_1^{2*} = \sum_i (r_i - n_i\hat{P}_i)^2/n_i\hat{P}_i^*(1 - \hat{P}_i^*).$$

The trial value for which $\chi_2^2 - \chi_1^{2*}$ equals 5.412 is the lower limit on the safe dose for the assay considered. With k dose levels, there will be k sets of assay data and k lower limits, the maximum of these limits being the one selected. (The likelihood ratio may be taken as an alternative criterion for setting limits; *see 10.*)

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Lysis of Homologous Cells by Sensitized Lymphocytes in Tissue Culture [1, 2]

WERNER ROSENAU, *M.D.*, *and* HENRY D. MOON, *M.D.*, *Department of Pathology and Cancer Research Institute, University of California, San Francisco Medical Center, San Francisco, California*

SUMMARY

The effects of sensitized lymphocytes of BALB/c mice on homologous cells of the L strain, originally derived from a C3H mouse, were studied in a synthetic tissue-culture medium. The reaction between sensitized lymphocytes and the sensitizing homologous cells was characterized initially by a clustering of lymphocytes around the L cells. This was followed by marked and progressive cytopathogenic changes of the L cells, which finally resulted in destruction of most of these cells. The cytopathogenic and cytolytic effects occurred in the absence of complement. There was no demonstrable antibody bound to the L cells, as determined by the fluorescent-antibody technique. The serums of the sensitized mice did not contain antibodies demonstrable by precipitation, immunodiffusion, or fluorescent-antibody techniques. These observations demonstrated that cytolysis of homologous cells by sensitized lymphocytes occurs *in vitro* in the absence of classical humoral antibodies and further emphasized the importance of cellular phenomena in immune processes.—J. Nat. Cancer Inst. 27: 471–483, 1961.

CELLULAR IMMUNITY is considered the most important mechanism for the rejection of homologous tissues on transplantation—humoral antibodies generally having little or no role in this process. This concept is based on the following evidence: failure of transfer of immunity to homologous grafts by serum (*1*), inconstant appearance of humoral antibodies in immune recipients, transfer of immunity to homologous grafts by lymph nodes and splenic tissue (*1*, *2*), and survival of homologous grafts in immunized hosts when enclosed in diffusion chambers (*3*). Transfer of cellular immunity by living cells has been successful only within the same species; in man, transfer with extracts of cells has been reported recently (*4*). A number of investigators working with sensitized lymphoid tissue and homologous explants *in vitro* failed to produce cytolysis (*5–9*); only Govaerts (*10*) has reported a destructive effect of lymphocytes on renal cells. Although most of our knowledge concerning

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the rejection of homografts has been obtained by experiments *in vivo*, it is difficult to obtain information about mechanisms of the immune reaction at a cellular level in the intact animal. Moreover, the role of additional factors such as complement cannot be critically evaluated.

The present studies were concerned with the effect of lymphocytes from sensitized animals on homologous cells in a synthetic tissue-culture medium; morphologic changes and cell counts were used as criteria. The serums of the sensitized animals were examined for antibodies by precipitation, immunodiffusion, and fluorescent-antibody techniques.

METHODS

Tissue-culture cells.—Cells of the L strain (clone A 2071-156-408B), originally isolated by Earle (*11*) from connective tissue of a male C3H mouse, were grown in milk dilution bottles in 90 percent medium 199 [3] (*12*) and 10 percent ox serum. All media employed in this study contained penicillin (100 μ/ml) and streptomycin (100 μg/ml). The cells were removed from the glass surfaces of milk dilution bottles by 0.0125 percent trypsin,[4] washed, and resuspended in medium 199 for injection.

Sensitization of mice.—Highly inbred female BALB/c mice, 10 weeks old, received intraperitoneal injections of a suspension of approximately 5 million living L cells on two occasions 4 weeks apart. This was followed by a third injection 4 weeks later directly into the spleen with the abdomen opened under barbiturate anesthesia. For this injection a volume of 0.05 ml containing approximately 2 million living cells was employed. The intrasplenic route of injection was used because lymphoid tissue close to the site of homologous transplants is known to develop a greater degree of sensitization (*2*).

For histopathologic studies, 36 of the mice receiving intraperitoneal injections were used. Some of these were killed at daily intervals, 1 to 11 days after the first injection, and the others, 3 and 4 days after the second. The omentum and peritonealized abdominal organs were removed and fixed in formalin. Paraffin sections were prepared and stained with hematoxylin and eosin.

Lymphocyte suspensions.—Four days after the intrasplenic injection, 6 of the sensitized mice were killed by fracture of the cervical spine and the spleens were removed at once under aseptic conditions. The spleens from 6 untreated mice were used as controls. The spleens were macerated with scissors, placed in medium 199, and the tissue fragments were broken up further by being pipetted vigorously. The resulting suspension was filtered through 2 layers of gauze, washed 3 times, and resuspended in medium 199 with a final concentration of 2 million nucleated splenic cells/ml. Although the suspension contained some red cells, over

[3] Microbiological Associates, Bethesda, Md.
[4] "Tryptar," Armour Pharmaceutical Co., Kankakee, Ill.

95 percent of the nucleated cells were lymphocytes, so the suspension will be referred to as a suspension of lymphocytes.

Lymphocytes and homologous cells in vitro.—Trypsinized and washed L cells (100,000) were grown in Leighton tubes in 1 ml of 90 percent medium 199 and 10 percent ox serum for 24 hours to allow the cells to attach to the glass. The ox serum had been previously inactivated by being heated for 30 minutes at 56° C. The medium was discarded, the tubes were rinsed with Hanks' solution, and to each tube 1 ml of the lymphocyte suspension in medium 199 without ox serum was added. A mixture of air (95%) and CO_2 (5%) was introduced into all tissue cultures and cultures were incubated at 37° C.

For morphologic studies, coverslips were placed into some of the Leighton tubes. For determination of the number of L cells, Leighton tubes without coverslips were used.

Four tubes were prepared for serial morphologic observations and 6 tubes for cell counts for each of the experimental and control mice. Additional tubes with coverslips from 1 control mouse and 2 experimental mice were prepared for fluorescent-antibody studies. A control group of L cells in medium 199 without lymphocytes was also included.

Morphology and cell counts.—Morphologic observations were carried out on coverslips, which were removed at 18, 40, and 48 hours, fixed in ether alcohol, and stained with hematoxylin and eosin. To determine the number of L cells at 48 hours, the culture medium was discarded, the tubes were rinsed gently with Hanks' solution, and 1 ml of 6 percent citric acid was added. After 1 hour of incubation at 37° C, the cytoplasm of the L cells had been digested and a suspension of nuclei had been obtained. One ml of 0.1 percent crystal-violet dye was added to each tube, and the nuclei of the L cells were counted in a standard hemocytometer. The L cell nuclei were large, oval, and vesicular and could be readily differentiated from the small, round, hyperchromatic nuclei of the lymphocytes. Since less than 1 percent of the L cells were multinucleated, the number of nuclei approximated closely the actual number of cells.

Fluorescent-antibody technique.[5]—Studies were conducted to examine the possible role of humoral antibodies in the interaction between L cells and lymphocytes. Coverslips with L cells exposed for 48 hours to lymphocytes were rinsed in phosphate-buffered saline, air-dried, and fixed in cold acetone. The preparations were dried, rehydrated, and treated with fluorescein-conjugated rabbit antimouse serum, which had been absorbed with beef cardiac powder. After the cover glasses were washed, they were mounted in glycerin and examined under a fluorescence microscope with a darkfield condenser and a mercury lamp. Additional coverslip preparations were counterstained with rhodamine to decrease nonspecific fluorescence; others were treated with fluorescein-conjugated sheep anti-rabbit serum as further controls to establish specificity of any fluorescence.

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[5] We gratefully acknowledge the advice and assistance of Dr. Barbara McIvor in the fluorescent-antibody studies.

Serologic procedures.—Antigen was prepared from washed L cells ground at 0° C in a mortar and suspended in saline. Pooled serums, obtained separately from control and experimental mice, were tested for the presence of humoral antibody by precipitin ring tests and immunodiffusion tests on cellulose-acetate paper (*13*). The serums were also added to L cells and tested by the usual fluorescent-antibody technique. Fluorescent rabbit antimouse serums, with and without rhodamine counterstaining, and fluorescein-conjugated sheep antirabbit serum, an additional control, were also used. These serologic methods were selected on the basis of their applicability to the study of unfractionated tissue preparations.

RESULTS

Reaction of Lymphocytes and Homologous Cells *In Vitro*

In serial examinations of the lymphocytes and L cells in medium 199 it was observed that the sensitized lymphocytes clustered in increasing numbers about the L cells and came into direct contact with these cells. This process became progressively more prominent and by 18 hours was very striking (fig. 2). The L cells began to withdraw their processes and rounded up, and the cytoplasm was quite eosinophilic. The cell borders became irregular, intracytoplasmic vacuoles appeared, and the cytoplasm started to fragment. The nucleus frequently became more dense and the chromatin clumped. Moderate numbers of cells had disintegrated by this time. Most cells were destroyed between 18 and 40 hours (figs. 4 and 5). There were occasional L cells surrounded by a few lymphocytes, but no degenerative changes were evident. A few L cells had no lymphocytic clustering.

The control lymphocytes showed no tendency to group about the L cells at 18 hours (fig. 1) but at 40 and 48 hours occasional L cells were surrounded by a few lymphocytes. Rare cytopathogenic changes involving the L cells were seen at this time but were of a markedly lesser degree and extent than those of the L cells exposed to sensitized lymphocytes. However, most of the L cells exposed to control lymphocytes remained unaltered (fig. 3). The L cells exposed to medium 199 alone showed no significant morphologic changes.

It should be emphasized that all of these studies of the reaction between lymphocytes and L cells were carried out in the absence of complement.[6]

Cell Counts

The morphologic evidence of a cytopathogenic effect was paralleled by a very significant difference in cell counts between the control and experimental groups. In all tubes the L cells exposed to sensitized lymphocytes

[6] In other studies the addition of guinea-pig complement and serum did not enhance the cytolytic effect. Although cytolysis was also demonstrable in a medium containing either guinea-pig complement (100 Kolmer units/ml) and 10 percent ox serum or 10 percent ox serum alone, the effect was not quite as striking as that seen in a protein-free medium.

showed a very significant decrease in numbers as compared to the controls (table 1). There was no significant difference between the L cells in cultures of nonsensitized lymphocytes and the L cells in media without lymphocytes.

TABLE 1.—Effect of lymphocytes on L cells *in vitro* at 48 hours

Groups	Donor mice	Number of L cells 1000/ml* ±	SE
L cells without lymphocytes	—	25. 7	2. 9
L cells and control lymphocytes	1	39. 1	2. 3
	2	38. 7	2. 7
	3	27. 7	2. 9
	4	35. 8	5. 6
	5	36. 0	3. 1
	6	27. 7	1. 6
L cells and sensitized lymphocytes	7	5. 6	0. 7
	8	3. 1	1. 8
	9	5. 2	0. 9
	10	4. 5	0. 4
	11	4. 1	0. 9
	12	5. 8	0. 8

* Average of 6 tubes.

Fluorescent-Antibody Studies of L Cells Showing Cytopathogenic Changes

The L cells exposed to control and sensitized lymphocytes showed no evidence of specific fluorescence after the application of fluorescein-conjugated antimouse serum. The L cells that were surrounded by sensitized lymphocytes did not show any specific fluorescence of cell membrane, cytoplasm, or nucleus. Rarely, a disintegrated cell remnant contained a few fluorescent granules of doubtful significance. Frequently the control or sensitized lymphocytes showed fluorescence.

Serums of Donor Animals

The pooled serums of the control and sensitized mice did not show any demonstrable antibody by either the precipitin ring or the immunodiffusion technique. L cells exposed to these serums did not show any specific fluorescence when examined by the double-layer fluorescent-antibody technique.

Homologous Cells *In Vivo*

The L cells injected intraperitoneally into mice appeared as very small implants on the peritoneal surfaces. Initially they were composed of numerous, compactly arranged, elongated cells with oval nuclei; mitoses were numerous. Vascularization of the implants occurred approximately 3 days after injection. By the 5th day marked lymphocytic infiltration of the implants was evident (fig. 6), frequently as perivascular cuffs. Thereafter, destruction of the implants occurred rapidly and was usually

complete by the 7th day after injection. On reinjection of L cells into mice that previously had rejected these cells, destruction occurred in less than 3 days. The injection of L cells into the spleens of previously sensitized mice resulted in marked splenic enlargement due to lymphoid hyperplasia and rapid destruction of the L cells.

DISCUSSION

The interaction between sensitized lymphocytes and homologous cells in a synthetic tissue-culture medium resulted in marked cytopathogenic changes and lysis of the homologous cells.

The immunologic mechanisms in this reaction apparently differed from those of the classical cytolytic humoral antibody system in that complement was not essential for the reaction, nor could an antibody bound to L cells be demonstrated by the fluorescent-antibody technique. In related studies the addition of guinea-pig complement and serum to the synthetic medium did not enhance the cytolytic effect. Indeed, in the presence of complement and serum the cytopathogenic changes were sometimes less striking and more variable, probably due to a nonspecific protective effect of protein on cells in tissue culture. Further evidence to support this hypothesis of a different mechanism was the absence of demonstrable antibodies in the serums of sensitized mice when examined by precipitin and immunodiffusion and fluorescent-antibody techniques.

The clustering of sensitized lymphocytes about the homologous cells was a most striking phenomenon, whereas this reaction was virtually absent with controls. Close contact between sensitized lymphocytes and homologous cells seems essential for the cytolytic reaction. This is also demonstrated by the survival of homografts *in vivo* when protected by diffusion chambers, as shown by Algire *et al.* (*3*) and Weaver *et al.* (*7*). Thus, the unsuccessful attempts by other investigators (*5–9*) to produce cytolysis *in vitro* with tissue fragments may be explained by the inadequate contact developed between the lymphocytes and the homologous cells.

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PLATE 54

FIGURE 1.—Control lymphocytes and L cells, 18 hours. No lymphocytic clustering; L cells do not show cytopathogenic changes. Hematoxylin and eosin. × 300

FIGURE 2.—Sensitized lymphocytes and L cells, 18 hours. Marked clustering of lymphocytes about L cells, which show early cytopathogenic alterations. Hematoxylin and eosin. × 300

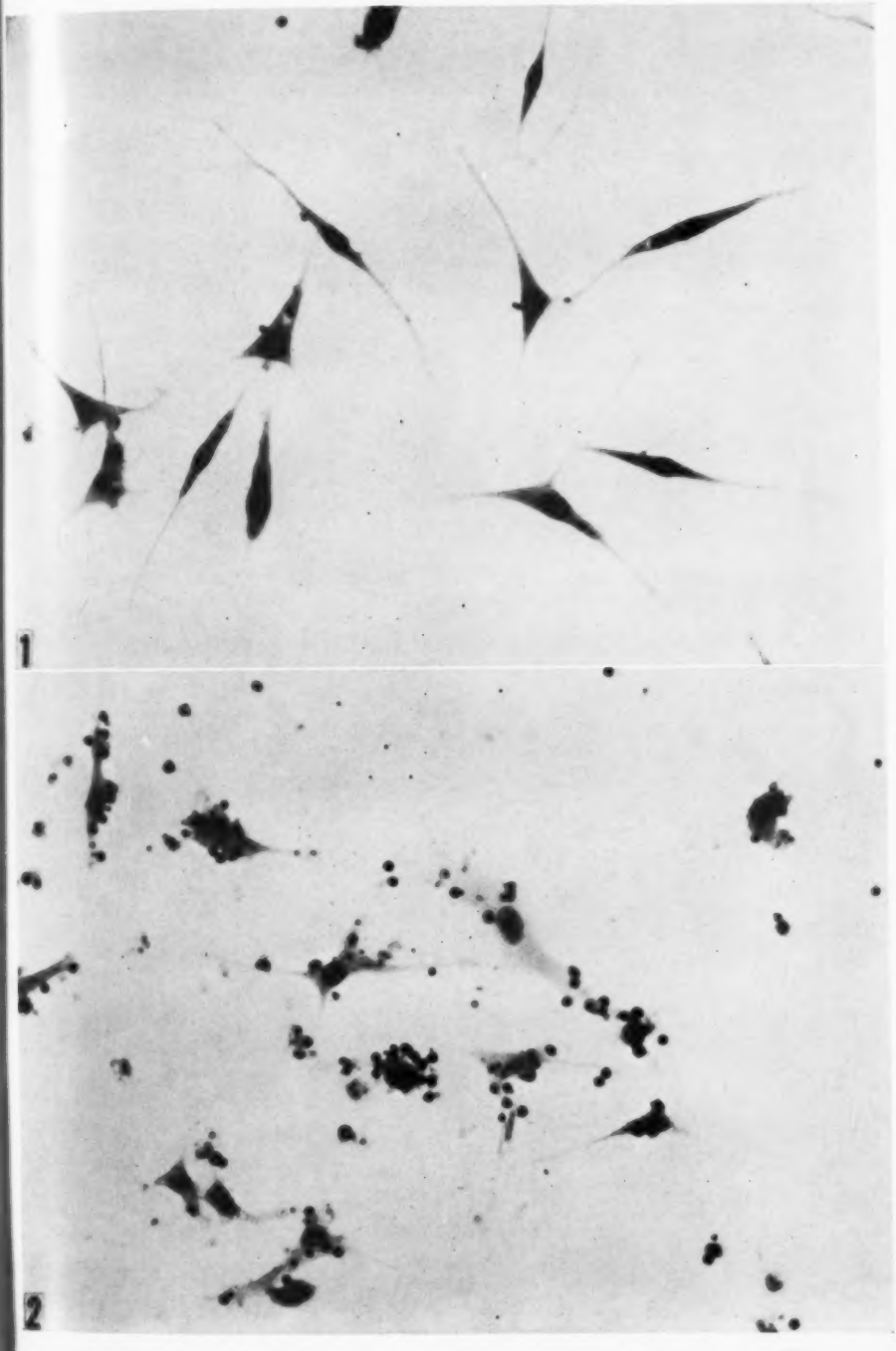

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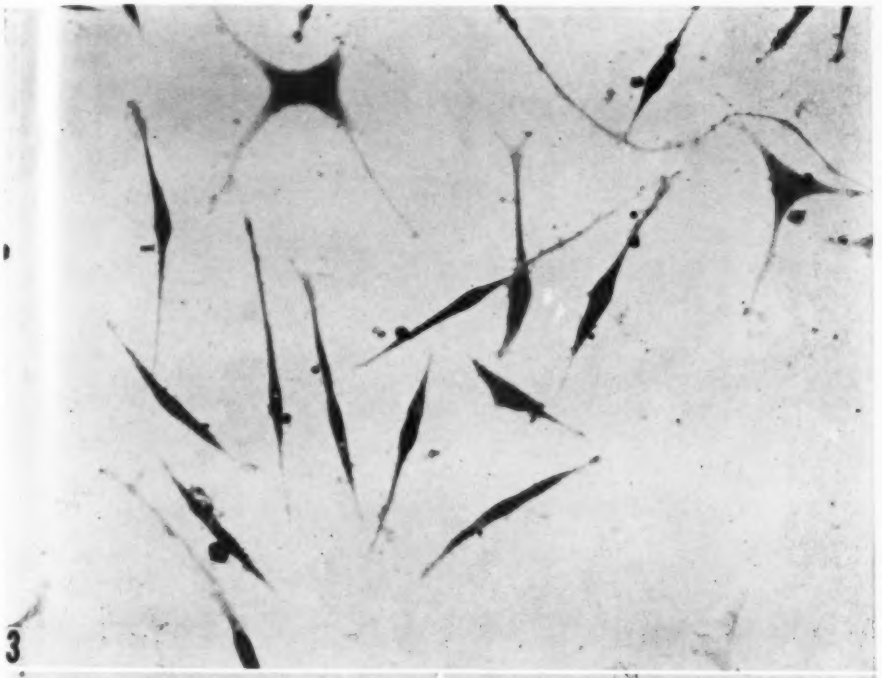
PLATE 55

FIGURE 3.—Control lymphocytes and L cells, 48 hours. No cytopathogenic changes of the L cells. Hematoxylin and eosin. × 300

FIGURE 4.—Sensitized lymphocytes and L cells, 48 hours. Only one surviving L cell surrounded by lymphocytes; all other cells have been lysed. Hematoxylin and eosin. × 300

FIGURE 5.—Sensitized lymphocytes and L cells, 48 hours. Complete destruction of all L cells. Hematoxylin and eosin. × 300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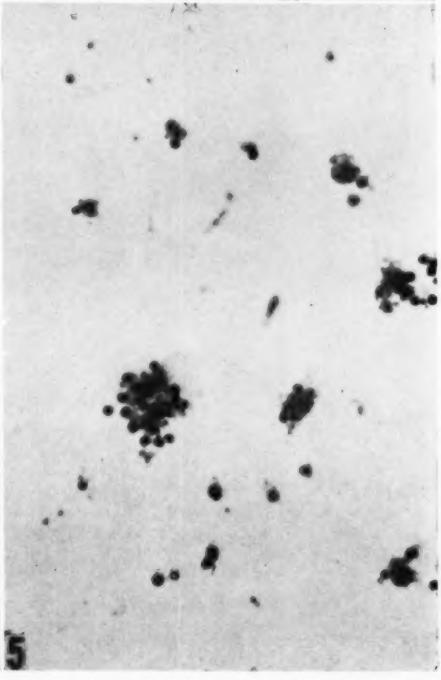
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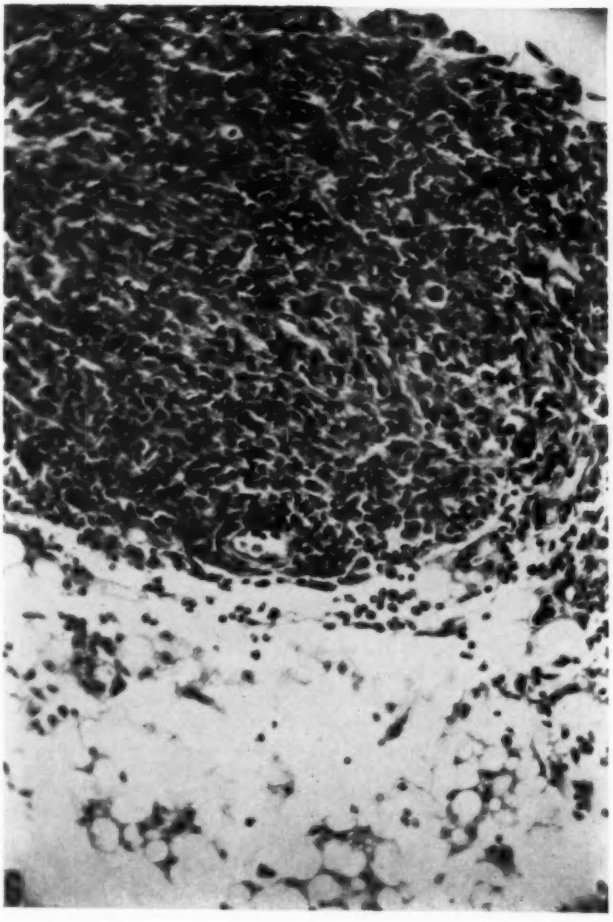
PLATE 56

FIGURE 6.—Peritoneal implant of L cells in BALB/c mouse showing vascularization and lymphocytic infiltration 5 days after injection of L cells. Hematoxylin and eosin. × 300



Announcements

Council for Medical Communication Institute

The Institute for Advancement of Medical Communication, with headquarters at 33 East 68th Street, New York 21, New York, has formed a Scientific Council of 20 scientists and educators to guide its program of research, development, and training aimed at improving communication among biomedical scientists and health science practitioners.

NCI Monograph No. 6

The sixth National Cancer Institute Monograph, *End Results and Mortality Trends in Cancer*, is scheduled for publication late in September, 1961.

Part I of the Monograph is: "End Results in Cancer." Tables give the 5- and 1-year survival rates for white cancer patients by primary site, age, first course of therapy, stage of disease at diagnosis, and sex; all registries combined, 1950–57. The papers in the section on "Special Analyses of End Results in Cancer" include: "Trends in Survival Rates of Cancer Patients: Connecticut and California," by Lillian M. Axtell, Lester Breslow, and Henry Eisenberg; "Survival Experience of Patients with Malignant Melanoma of the Skin, 1950–57," by Herman Heise and Edward T. Krementz; "Survival Experience of Patients with Cancer of the Tongue," by Fred Ederer and N. Henry Moss. The section on "Methodology of Survival Analysis in Cancer" contains: "The Relative Survival Rate: A Statistical Methodology," by Fred Ederer, Lillian M. Axtell, and Sidney J. Cutler; "A Note on Follow-up of Lost Patients," by John C. Bailar III, Ruth Lowry, and Ira S. Goldenberg.

Part II, "Cancer Mortality Trends in the United States, 1930–55," by Tavia Gordon, Margaret Crittenden, and William Haenszel, describes the major trends in cancer mortality and the primary demographic differentials. Essentially, this report is designed to provide a framework of reference for cancer investigators working with data for this country and as a counterpart to the systematic descriptions of cancer mortality available for other countries. The substance of Part II will be found in the Appendix tables, which present mortality data by specific cancer sites for the United States.

Errata

Volume 26, Number 4, April 1961

In the paper "Liver Enzymes During Hepatic Carcinogenesis," by Trams, Inscoe and Resnik, page 966, legend for text-figure 4, *for* "The amounts of kynurenine formed by animals on Purina chow diet varied from 19.8 to 22.9 μmoles/g wet-liver weight hour" *read* ". . . varied from 1.98 to 2.29. . . ."

Volume 26, Number 5, May 1961

In the paper "Sarcomatous Change and Maintenance of Differentiation in Long-Term Cultures of Mouse Mammary Carcinoma," by Sanford *et al.*, page 1144, line 5, *for* "Strains 1352 and 2940 failed to grow in mice . . ." *read* "Strain 1352 failed to grow in mice. . . ."

Page 1148, under "Experiments on Cell Strains 2848 and 2940," line 20, *for* "Cells of strain 2940 . . . failed to grow when implanted into 2 irradiated mice after 49 weeks of culture (table 1)" *read* "Cells of strain 2940 . . . grew as a type-B tumor in one irradiated mouse after 21 weeks of culture (table 1)."

Page 1149, line 4, *for* "One type resembled the primary carcinoma cell, though limited efforts to transplant the cells into mice were unsuccessful," *read* "One type resembled the primary carcinoma cell and grew as type-B tumor when transplanted after 21 weeks of culture."

Manuscripts Approved for JNCI, June 15 to July 15, 1961

"Comparative study of the carcinogenic activity of nitrosamines," by Mary F. Argus and Cornelia Hoch-Ligeti.

"Studies on the exchange of fluids between host and tumor. I. A method for growing 'tissue-isolated' tumors in laboratory animals," by Pietro M. Gullino and Flora H. Grantham.

"Persistent Theiler's virus in ependymoma tissue culture and the problem of virus-like bodies seen by electron microscopy," by Harold E. Pearson and Richard F. Baker.

"Distribution of primary lung carcinomas in relation to time as determined by histochemical techniques," by Doris L. Herman and Margaret Crittenden.

"Experimental mammary adenocarcinoma of rats," by Harry Shay, Margot Gruenstein, and Woodrow B. Kessler.

"Influence of adrenocortical, androgenic, and anabolic hormones on the development of carcinoma and cirrhosis of the liver in A × C rats fed *N*-2-fluorenyldiacetamide," by Harlan I. Firminger and Melvin D. Reuber.

"Glucose metabolism of Rous sarcoma virus-induced tumors. II. Isotope studies of alternate pathways," by James Ashmore, George Weber, Gouri Banerjee, and William C. Love.

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